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ELECTRON MICROSCOPE STUDIES
ON THE MULTIPLICATION OF A CANINE ADENOVIRUS
BY MEANS OF ENZYME CYTOCHEMISTRY
AND HIGH RESOLUTION AUTORADIOGRAPHY

by



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A THESIS

Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
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FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, "Electron Microscope Studies on the Multiplication of a Canine Adenovirus by Means of Enzyme Cytochemistry and High Resolution Autoradiography" submitted by Mahmood Shamsi Shahrabadi, in partial fulfillment of the requirements for the degree of Master of Science in Microbiology.

ABSTRACT

The virus of canine laryngotracheitis, like most other adenoviruses, manifested certain nuclear changes in the process of multiplication. These changes were further studied to determine the nature of the inclusions by specific enzyme digestion and autoradiography.

The earliest change in the infected cell was the appearance of granular inclusions in the nucleus. These inclusions which appeared by 12 hr after infection were partially digested with proteolytic enzymes. Following treatment with proteolytic enzymes, digestion with DNase resulted in the complete removal of these bodies. Furthermore, the incorporation of tritiated thymidine and leucine in these structures indicated that they were composed of nucleoprotein. The DNA content of the early inclusions incorporated into the virus particles, suggesting that these bodies contained viral DNA.

The early inclusions increased in size and appeared as ring form inclusions by 16 hr after infection. These inclusions had the same degree of sensitivity to digestion with the enzymes as the early inclusions and were similarly labelled with tritiated thymidine and leucine. The rapid incorporation of tritiated uridine into the early and the ring form inclusions indicated the presence of RNA in these structures and led to the conclusion that they were the site of viral RNA synthesis. By 18 hr after infection, the ring form inclusions merged to form

a single large inclusion in which large groups of virus particles were seen. At this time, dark and light staining inclusions were observed in the infected cells. The dark staining inclusions appeared both in the nucleus and in the cytoplasm. On the basis of their sensitivity to proteolytic enzyme digestion and labelling with tritiated leucine, it was concluded that they were composed of protein(s).

The light staining inclusions which appeared in the nuclei of infected cells, were digested with proteolytic enzymes. The incorporation of prelabelled proteins into these bodies indicated that they were formed from the accumulation of some proteins synthesized during the infection. At the late stage of infection (18 hr), the nucleoli of infected cells were labelled with tritiated uridine, suggesting continuous RNA synthesis in these organelles. In infected cells, host DNA marginated toward the nuclear membrane and did not appear to be a precursor for the formation of inclusions and virus particles.

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TABLE OF CONTENTS

	<u>Page</u>
Title Page.....	i
Approval Sheet.....	ii
Abstract.....	iii
Acknowledgements.....	v
List of Tables.....	ix
List of Figures.....	x
List of Abbreviations.....	xiv
<u>INTRODUCTION</u>	1
Light Microscope Studies.....	2
Electron Microscope Studies.....	5
Biochemical Studies.....	7
Histochemical Studies.....	10
Autoradiography Technique.....	13
<u>MATERIAL AND METHODS</u>	16
Virus.....	16
Cell Culture.....	16
Tissue Culture Media.....	16
Virus Production.....	16
Virus Assay.....	17
Electron Microscopy.....	18
Shadowing.....	21
Digestion of thin sections with Enzymes.....	21
Autoradiography.....	22
Incorporation of radioactive precursors.....	22

<u>MATERIAL AND METHODS</u> , continued	<u>Page</u>
Tritiated thymidine incorporation.....	22
Incorporation of tritiated uridine.....	24
Incorporation of tritiated leucine.....	24
Estimation of tritiated uridine incorporation into RNA and DNA.....	25
Preparation of the specimen and application of the emulsion.....	26
Exposure time.....	29
Development.....	29
Staining.....	29
<u>RESULTS</u>	32
Plaque assay of ICL virus.....	32
Electron microscopy.....	34
Fine structure of uninfected MDC cell.....	34
Appearance of early inclusions in infected cells...	35
Development of early inclusions into spheres.....	35
Further development of the ring form inclusions and the appearance of dark staining inclusions...	36
Appearance of light staining inclusions.....	37
Cytochemical studies.....	42
Digestion with proteolytic enzymes.....	42
Treatment of thin sections with RNase and DNase....	42
The appearance of uninfected and early infected cells after enzyme digestion.....	43
Digestion of dark and light staining inclusions....	44
Digestion of virus particles.....	45
Electron microscope autoradiography of thin sections.....	56

<u>RESULTS</u> , continued	<u>Page</u>
Location of tritiated thymidine.....	56
Location of tritiated uridine.....	65
Location of tritiated leucine.....	74
<u>DISCUSSION</u>	81
The nature of the early and ring form inclusions...	82
Composition of the early and ring form inclusions..	82
Nature of DNA present in the inclusions.....	84
The nature of the protein present in the early and ring form inclusions.....	86
Presence of RNA in the inclusions.....	88
The nature of the dark and light staining inclusions.....	91
Margination of host DNA.....	95
<u>BIBLIOGRAPHY</u>	96

LIST OF TABLES

<u>Table No.</u>		<u>Page</u>
I.	The Effect of Incubation Time on the number of plaques.	33
II.	Incorporation of ³ H-Uridine into RNA and DNA.	68

LIST OF FIGURES

<u>Figure</u>	<u>Facing Page</u>
1. Electron micrograph of Ilford L-4 crystals in thin layer.....	31
2. Electron micrograph of uninfected MDC cell...	38
3. Electron micrograph of MDC cell 12 hr after infection with ICL virus.....	39
4. Electron micrograph of MDC cell 16 hr after infection with ICL virus.....	39
5. Electron micrograph of MDC cell 16 hr after infection with ICL virus.....	40
6. Electron micrograph of MDC cell 18 hr after infection with ICL virus.....	40
7. Electron micrograph of MDC cell 20 hr after infection with ICL virus.....	41
8. Electron micrograph of dark staining inclusions.....	42
9. Electron micrograph of MDC cell digested with pronase.....	47
10. Electron micrograph of 12 hr infected cell digested with pronase.....	47
11. Electron micrograph of 12 hr infected cell digested with trypsin and DNase.....	48
12. Electron micrograph of 12 hr infected cell digested with RNase.....	48
13. Electron micrograph of 16 hr infected cell digested with trypsin.....	49
14. Electron micrograph of 16 hr infected cell digested with trypsin and RNase.....	49
15. Electron micrograph of 16 hr infected cell digested with trypsin and DNase.....	50
16. Electron micrograph of 20 hr infected cell digested with pronase.....	50

<u>Figure</u>	<u>Facing Page</u>
17. Electron micrograph of 20 hr infected cell treated with distilled water.....	51
18. Electron micrograph of 20 hr infected cell digested with DNase.....	51
19. Electron micrograph of 20 hr infected cell digested with trypsin and shadowed with paladium.....	52
20. Electron micrograph of 20 hr infected cell shadowed with paladium.....	52
21. Electron micrograph of ICL virus in MDC cell digested with trypsin.....	53
22. Electron micrograph of ICL virus in MDC cell digested with trypsin and DNase.....	53
23. Electron micrograph of ICL virus in MDC cell digested with trypsin and DNase.....	54
24. Electron micrograph of 20 hr infected cell digested with RNase.....	55
25. Electron micrograph of 20 hr infected cell digested with pronase and RNase.....	55
26. Autoradiograph of noninfected cell labelled with tritiated thymidine.....	60
27. Autoradiograph of 12 hr infected cell labelled with tritiated thymidine.....	60
28. Autoradiograph of 16 hr infected cell labelled with tritiated thymidine.....	61
29. Autoradiograph of 19 hr infected cell labelled with tritiated thymidine.....	61
30. Autoradiograph of 19 hr infected cell labelled with tritiated thymidine and digested with pronase.....	62
31. Autoradiograph of 19 hr infected cell labelled with tritiated thymidine and digested with pronase and DNase.....	62

<u>Figure</u>	<u>Facing Page</u>
32. Autoradiograph of infected cell labelled with tritiated thymidine and treated with distilled water.....	62
33. Autoradiograph of 12 hr infected cell labelled with tritiated thymidine.....	63
34. Autoradiograph of infected cell labelled with tritiated thymidine and chased for 7 hr.	63
35. Autoradiograph of MDC cell labelled with tritiated thymidine.....	64
36. Autoradiograph of prelabelled cell with tritiated thymidine and infected with ICL virus.....	64
37. Autoradiograph of tritiated thymidine labelled cell after a long exposure time.....	64
38. Autoradiograph of prelabelled infected cell after a long exposure time.....	64
39. Autoradiograph of noninfected cell labelled with tritiated uridine.....	69
40. Autoradiograph of noninfected cell labelled with tritiated uridine.....	69
41. Autoradiograph of 12 hr infected cell labelled with tritiated uridine.....	70
42. Autoradiograph of 12 hr infected cell labelled with tritiated uridine.....	70
43. Autoradiograph of 16 hr infected cell labelled with tritiated uridine.....	71
44. Autoradiograph of 18 hr infected cell labelled with tritiated uridine.....	71
45. Autoradiograph of 18 hr infected cell labelled with tritiated uridine.....	72
46. Autoradiograph of infected cell labelled with tritiated uridine and digested with RNase	72
47. Autoradiograph of infected cell labelled with tritiated uridine and digested with pronase and RNase.....	73

<u>Figure</u>	<u>Facing Page</u>
48. Autoradiograph of noninfected cell labelled with tritiated uridine and digested with pronase and RNase.....	73
49. Autoradiograph of 16 hr infected cell labelled with tritiated leucine.....	77
50. Autoradiograph of 18 hr infected cell labelled with tritiated leucine.....	77
51. Autoradiograph of 18 hr infected cell labelled with tritiated leucine.....	78
52. Autoradiograph of 18 hr infected cell labelled with tritiated leucine.....	78
53. Autoradiograph of tritiated leucine labelled cell digested with trypsin.....	79
54. Autoradiograph of 18 hr infected cell prelabelled with tritiated leucine.....	79
55. Autoradiograph of infected cell labelled with tritiated leucine and chased for 6 hr...	80
56. Autoradiograph of infected cell labelled with tritiated leucine and chased for 6 hr...	80
57. Autoradiograph of infected cell labelled with tritiated leucine and chased for 6 hr...	80

LIST OF ABBREVIATIONS

c	-curie
uc	-microcurie
DKL	-dog kidney line
MDC	-Madin-Darby Canine kidney
ICL	-Infectious canine laryngo- tracheitis
TCA	-trichloroacetic acid
DNase	-deoxyribonuclease
RNase	-ribonuclease
mRNA	-messenger RNA
GMA	-glycol methacrylate
MEM	-minimal essential medium
HLA	-Hank's lactalbumin medium
HBSS	-Hank's balanced salt solution
P.F.U.	-plaque forming unit
PBS	-phosphate buffered saline

INTRODUCTION

Adenoviruses present several features that make them important to study from the medical and biological points of view.

The group includes about 45 different types, some of which can cause a variety of diseases in man and animals. In addition, they are of particular interest because some members are oncogenic.

The nature of the nuclear reproduction of these viruses promotes the study of the developmental features occurring during reproduction. Extrapolation of information from the study of adenoviruses may be useful in other viruses which demonstrate similar stages in multiplication. The explanation of the accumulation of large quantities of material appearing as inclusions may open the way for better understanding of viral assembly in adenoviruses as well as other viruses.

The infectious canine laryngotracheitis (ICL) virus, isolated from dogs with a respiratory disease, has been shown to be serologically related to the adenovirus group and designated as A26/61 (Ditchfield et al, 1962). Further studies by Yamamoto (1963, 1966, 1967), Gaunt (1966), and Yamamoto and Marusyk (1968), have shown that its growth characteristics and physico-chemical properties are similar to those of other adenoviruses.

Electron microscopic examination of purified virus

particles (Yamamoto and Marusyk, 1968) using the phosphotungstic acid negative contrast staining procedure revealed that the particles were icosahedra of 80 m μ in diameter, composed of 252 capsomers consisting of hexons, pentons, and 35 m μ long fibres attached to the pentons. These morphological properties are the major characteristics of the adenovirus group (Andrew et al, 1961).

Electron microscopic examination of thin sections of infected cells has shown that adenoviruses multiply in the nuclei of host cells (Kjellen et al, 1955, Morgan et al, 1956).

Light microscope studies

The sequential changes in nuclei of infected cells during virus multiplication has been studied by Boyer et al (1957). These authors differentiated the various types of human adenoviruses on the basis of the alterations produced in the nuclei of HeLa or human amniotic infected cells.

I. A group of adenoviruses which produced eosinophilic inclusions 14 to 16 hr after virus inoculation. These inclusions initially were Feulgen-negative and 2 to 3 hr later developed granular Feulgen-positive basophilic cores. They eventually formed a dense basophilic mass which was probably composed of virus crystals. This group includes adenovirus types 1, 2, 5, and 6. The appearance of bar shaped eosinophilic crystals in the nuclei of infected cells with type 5 adenovirus was described by Morgan et al,

(1957). These inclusions, which were Feulgen-negative, did not appear in the nuclei of infected cells with adenovirus types 1, 2, and 6.

II. A group of adenoviruses (including types 3 and 4) which caused the formation of irregular, granular, eosinophilic masses and the development of a rarified zone beneath the nuclear membrane which was associated with rearrangement of chromatin into a network pattern. These alterations were the first events which could be detected 14 hr after infection. Later on, the central area of the enlarged nuclei became more intensely basophilic and consisted of either densely packed granules or a strikingly regular honey-comb. These coarse network type Feulgen-positive central masses were associated with the appearance of sharp-edged crystal-like structures which varied from eosinophilic and Feulgen-negative to basophilic and Feulgen-positive. In the later stage of infection, the central basophilic zone appeared to be formed of many small crystals which were shown to be composed of virus particles (Morgan et al, 1956, Harford et al, 1956). Ovoid or wedge-shaped compartments of rosette form nuclei were seen in some of the infected cells.

There was no detectable change in the cytoplasm of host cells during the replication cycle of these two groups of viruses.

Light microscope studies of primary dog kidney cells

infected with ICL virus by Yamamoto (in press, Microbios) showed that the nuclear alterations induced by this virus were slightly different from those described by Boyer et al, (1957) and by Ginsberg and Dingle (1965) for adenovirus types 1, 5, and 6. The appearance of basophilic inclusions in the nuclei of infected cells was the earliest morphological change which could be observed 12 hr after infection. These early inclusions were Feulgen-positive and increased in size to form spheres which appeared 16 hr after infection. The central part of these spheres consisted of eosinophilic material and showed the absence of DNA by the Feulgen reaction. The outer part, which appeared as a rim, was basophilic and Feulgen-positive. As the inclusions increased in size, the interior eosinophilic region became filled with DNA-containing material which was sensitive to deoxyribonuclease. The size of the nucleus markedly increased and reached the maximum at 28 hr after infection.

At 20 hr post infection, most of the nuclear space became occupied with an eosinophilic mass containing basophilic granules. In some cells, round brightly stained eosinophilic inclusions could be observed which were also found to be Feulgen-negative and were located either just inside the nuclear membrane or within the central nuclear area. In the late stage of infection, the nucleus appeared to be shrunken and full of homogeneous densely staining material. The whole cell became pycnotic and the nucleus

was indistinguishable from the cytoplasm. No change in the cytoplasm of the infected cells could be observed.

Electron microscope studies

Electron microscope studies by Yamamoto (1969) have shown the sequential changes of primary dog kidney cells infected with ICL virus. The penetration of virus particles into the cell was by a process akin to phagocytosis and was similar to the entry of adenovirus types 2 and 4 into HeLa cells (Dales, 1962). The ICL virus could be seen bound within a membrane in the cytoplasm 3 to 4 hr after infection. The entry into the nucleus of the virus either whole or as uncoated viral DNA was not clear.

Lawrence et al, (1967) indicated that within 60 minutes after adsorption of adenovirus type 5 to KB cells, 97% of the cell-associated virus was TCA-insoluble and deoxyribonuclease-sensitive. They reported that sensitivity of viral particles to deoxyribonuclease which occurred after penetration of the virus into the cells was due to the removal of some viral protein coat, and termed it "uncoating". Furthermore, the uncoated virus had a sedimentation coefficient value of 400S, whereas viral DNA and purified virus had values of 30S and 800S respectively. They concluded that the uncoated virus DNA was associated with some structural proteins, and further alterations must occur in the cytoplasm or in the nuclei before viral DNA replication commences.

The earliest alteration observable in the nuclei of

ICL infected cells was the granularity of the nucleus 8 hr after infection. These granules increased in number and formed irregular granular inclusions which appeared as dark staining areas by 12 hr post infection. The granular inclusions increased in size and the central part became light staining. By 16 hr after infection, the inclusions were observed as dark staining rings surrounding a lighter staining central part. The dark staining region expanded into the central core to form a uniformly dense inclusion with an undifferentiated centre. A few virus particles could be observed in the nucleus at this time of infection. In light microscope examination, the granular and ring form inclusions observed in the electron microscope appeared as early basophilic granules and spherical basophilic structures respectively (Yamamoto, in press, Microbios).

At 20 hr after inoculation, two kinds of distinct intranuclear inclusions could be visualized:

I. Circular or oval dark staining inclusions with a very dense granular structure were usually found close to the nuclear membrane. These inclusions appeared as brilliant eosinophilic spots when examined in the light microscope.

II. Light staining inclusions of irregular shape with a homogeneous structure associated with virus particles were located near the nuclear membrane. At this time, virus particles were seen to aggregate and later could be found as crystalline arrays. The release of virus from

the nucleus into the cytoplasm was by formation of protrusions from the nuclear membrane containing large numbers of virus particles. The membrane-bound virus were pinched off into the cytoplasm where the membrane was destroyed and the virus particles released.

It seems that the morphological changes in the nuclei of ICL virus infected cells are different from the alterations induced by other adenoviruses. Electron microscope studies of Hep 2 cells infected with adenovirus type 5 by Morgan et al, (1960) has shown the presence of large protein crystals in the nuclei of infected cells. The same observation has been reported recently by Weber and Stich (1969) in Hep 2 cells infected with an adenovirus type 2 strain. In contrast, none of these crystalline structures were observed by Yamamoto (1969) in ICL infected cells.

In 1967, Martinez-Palomo studied the multiplication of adenovirus type 12 in KB cells with the electron microscope. He found the appearance of four types of inclusions in the nuclei of infected cells. These inclusions which consisted of either protein or nucleoprotein appeared about 20 hr after infection and had no similarity to the inclusions formed in ICL infected cells.

Biochemical studies

Biochemical events which occur in infected cells during the multiplication cycle of virus have been investigated mostly on adenovirus type 2, 4, and 5. In 1965,

Polasa and Green found that the formation of an early protein(s) was necessary for the replication of adenovirus type 2 DNA in KB cells. The synthesis of this protein started 2 to 3 hr after infection and continued for 2 hr. This was the earliest virus-induced event which could be detected following the infection.

In 1959, Ginsberg and Dixon found that the synthesis of adenovirus type 4 DNA in HeLa cells started 10 to 11 hr post infection. Similar observations were reported by Flanagan and Ginsberg (1962) with adenovirus type 4. Further studies by Ginsberg et al, (1967) indicated that host DNA synthesis was inhibited at the time when viral DNA synthesis began. Recently, Hodge and Charff, (1969), studying the multiplication of adenovirus type 2 in synchronized HeLa cells reported that the synthesis of host DNA after infection was dependent on the stage of its life cycle at which the replication of viral DNA was initiated. When viral DNA was synthesized during G₁ phase, the replication of cellular DNA stopped completely. When viral DNA synthesis began after the onset of S phase, cellular DNA replication was partially, but not completely, inhibited.

An increase of RNA synthesis in adenovirus type 5 infected HeLa cells was found by Flanagan and Ginsberg (1964), who showed that RNA synthesis was essential for production of infectious particles. The synthesis of this RNA which was reported to be complementary to viral DNA, started

8 hr after infection, reaching its maximum at 14 hr and then decreased to a level slightly higher than the uninfected cells. The same results were obtained by Thomas and Green, (1966) with adenovirus type 2 infected cells, but the synthesis of viral RNA started at about 6 hr, with a maximum rate at 18 hr post infection.

Rose et al, (1965), by the hybridization method, showed that the increase of RNA synthesized in KB cells infected with adenovirus type 2 was viral specific mRNA. Recent studies by White et al, (1969) showed that the viral mRNA produced in HeLa cells infected with adenovirus type 2 was relatively stable with a functional half-life of about 6 hr.

In 1967, Ginsberg et al reported that host mRNA synthesis in KB cells infected with type 5 adenovirus continued until 28 hr post infection. However, there is no evidence available to show whether the host ribosomal RNA synthesis continues during infection.

Wilcox and Ginsberg, (1963) found that the synthesis of virus structural protein in HeLa cells infected with adenovirus type 5 commenced at about 11 hr after infection which was about 2 to 3 hr before virus maturation. Further studies by Thomas and Green, (1966) indicated that adenovirus type 2 coded proteins were made in the cytoplasm of KB cells. A similar result was obtained by Velicer and Ginsberg (1968) in KB cells infected with adenovirus type 5. They showed that the synthesis of viral capsid proteins

was accomplished in the cytoplasm of infected cells, then transferred to the nuclei for assembly into virus particles.

The effect of adenovirus infection on the synthesis of host-cell protein was studied by Bello and Ginsberg (1967). They indicated that protein synthesis of KB cells infected with adenovirus was inhibited about 16 hr after infection.

The discussion mentioned above were some of the biochemical events which have been studied in infected cells with human adenovirus; it is assumed that all adenoviruses have similar patterns of multiplication, although the exact times of these events may vary.

Histochemical studies

Histochemical techniques using different stains, such as acridine orange stain for differential staining of nucleic acids, and enzyme digestion of infected cells with virus have been applied by many investigators in the study of virus multiplication.

In 1964, Mayor studied the multiplication of adenovirus types 3 and 7 in monkey kidney cells using acridine orange staining technique and indicated an increase of RNA synthesis in the nucleoli 16 hr after infection. At 20 hr post infection, a bright staining ring of RNA surrounding the complete DNA containing inclusions was observed. At 24 hr, in many cells, RNA-staining material appeared not only as a ring around the viral inclusions, but also in the centre of the crystalline matrix. These rings of RNA were absent at 36 hr post infection.

The methods of embedding tissue for electron microscopy which are used most frequently, are embedding in Vestopal (Ryter and Kellenberger, 1958), Epon (Luft, 1961), and Araldite (Glauert et al, 1956). Preservation of the fine structure of tissue embedded in these media is satisfactory. However, they have the disadvantage of being water insoluble, and cannot be used effectively for enzyme histochemistry.

The introduction of water soluble embedding media (Rosenburg et al, 1960) has made it possible to improve electron microscope cytochemical studies because of the ability to digest with enzymes directly on thin sections. This method of digestion was used with the tissue fixed in aldehyde and embedded in water soluble epoxy resin, Durcupan, by Leduc and Bernhard (1960, 1961, 1962).

Selective digestion of proteins was obtained by trypsin and pepsin, but the tissue was unaffected by deoxyribonuclease and ribonuclease. They concluded that reactive groups in the specimen may combine with embedding medium and prevent the enzyme digestion. When they used glycol methacrylate (2-hydroxyethylmethacrylate) as embedding media, it was found that the tissue fixed in formaldehyde was digestible by both proteolytic enzymes and nucleases.

Glycol methacrylate (GMA) is completely miscible with water, ethanol and ether. The tissue is dehydrated by a series of graded solutions of GMA with water. The

polymerization of the methacrylate is a critical procedure, since in some capsules (which are used for embedding and polymerization), the polymerization is incomplete and non-homogeneous. Many methods have been applied to improve the conditions for polymerization: polymerization at high temperature, as 80°C - 90°C (Borysko, 1956), in a nitrogen atmosphere to prevent the inhibition effect of oxygen on polymerization (Moore and Grimley, 1957), by ultraviolet light at low temperature, -10°C (Muller, 1957, Sjostrand and Baker, 1958). Better results have been obtained by ultraviolet light polymerization than by the other methods.

The major difficulty is the problem of preserving tissue structure in water-soluble embedding media. Extensive distortion and extraction may occur in embedded tissue. It is believed that the destruction of the tissue organization is caused by irregular polymerization (Sjostrand, 1967).

The application of enzyme digestion in virology started in 1961 by Thomas and Williams who removed the core of ethanol-fixed, methacrylate-embedded Tipula irridescent virus by treating sections with trypsin and DNase. Epstein (1962) was able to digest the cores of extracellular adenovirus, Rous virus, after fixation in KMnO_4 , by nucleases. In 1962, Bernhard and Tournier reported that the core of formalin fixed reoviruses and adenoviruses embedded in GMA were susceptible to the

action of RNase and DNase, respectively.

Leduc and Bernhard (1967) were able to improve the method of embedding tissue in water-soluble material for enzyme cytochemistry and autoradiography studies. They showed that glutaraldehyde fixed tissue embedded in GMA was readily digestible by proteolytic enzymes and nucleases. Using this embedding procedure, Martinez-Palomo (1967) was able to digest the inclusions produced in adenovirus type 12 infected cells by treatment of the thin sections with proteolytic enzymes and nucleases. Dalton et al (1968) reported that the granules of Lymphocytic choriomeningitis virus fixed in glutaraldehyde and embedded in GMA were readily digestible by ribonuclease.

In this study, these combined developments, using GMA and glutaraldehyde, made it possible to obtain selective digestion of biological macromolecules in thin sections.

Autoradiography technique

Tritiated precursors have been used by a number of investigators for light microscopic autoradiography of cells infected with virus. However, this technique is not readily applicable to virological studies because the resolution is insufficient for viral particles to be visualized. Therefore, only limited information can be obtained as to whether viral particles and viral induced inclusions are labelled with the radioactive precursors under these conditions.

With electron microscope autoradiography, a much

higher resolution can be obtained and this allows the ability to correlate more accurately the pattern of labelling with the structures as seen by the electron microscope. Recently, methods of autoradiography with the electron microscope have reached a level of development that warrant general use in a variety of experimental situations.

Liquir-Milward (1956) was probably the first to couple autoradiography with electron microscopy. Since that time, improvements have been made both in nuclear emulsions and in the technique of their application to the specimen.

In 1962, Caro and Van Tubergen, working with Ilford L-4 emulsion, introduced a method in which a thin film of emulsion was picked up on a wire loop and placed over the specimen. Kochler, Muhlethaler and Frey-Wyssling (1963) used the centrifuge method for application of the emulsion over the sections. The membrane technique introduced by Salpeter and Bachmann (1965) has improved the method of preparing a thin layer of emulsion over the specimen. Furthermore, the introduction of new emulsions with small crystal size made it possible to obtain high resolution with electron microscope autoradiography (Granboulan, 1963).

This study was undertaken to characterize the nature of inclusions produced in a dog kidney cell line (MDC) following the infection of cells with the canine laryngo-

tracheitis virus and to determine their possible role in virus maturation.

MATERIALS AND METHODS

Virus

The canine adenovirus obtained from Dr. J. Ditchfield, University of Toronto, was provided by Dr. T. Yamamoto. The virus has been propagated on dog kidney cell line (DKL) and stored at -20°C.

Cell culture

Two kinds of cell culture were used in this study:

1. Madin-Darby canine kidney cell line (MDC), obtained from the American Type Culture Collection, Rockville, Md.
2. Primary dog kidney cells prepared by trypsin digestion of minced newborn dog kidneys. This cell culture was used for comparative assay of viral infectivity.

Tissue culture media

Commercial Eagle's (1959) minimal essential media (MEM) supplied by General Biochemical, Grand Island, New York, was used in most of the experiments. The media was supplemented with 5% calf serum and 100 I.U./ml of penicillin-G and 100 ug/ml of streptomycin-sulfate. The pH was adjusted to 7.4 with 7.5% sodium bicarbonate. Hank's (1949) balanced salt solution (HBSS) containing 0.5% lactalbumin hydrolysate (Difco) was used for the preparation of overlay medium for virus assay.

Virus production

Each Roux bottle containing complete monolayers of MDC cells was inoculated with 5 ml of virus suspension con-

taining 2×10^8 PFU. The virus was allowed to adsorb for 1.5 hr at 37°C with frequent agitation at 10 min intervals. After the adsorption period, 75 ml of fresh medium was added and incubated at 37°C until cytopathic effect was complete (3 days). The cells were harvested and centrifuged at 1500 rpm in a clinical centrifuge for 10 min. The pellet was resuspended in 7 ml of growth medium. The cells were disrupted by freezing and thawing 3 times. The cell debris was sedimented by centrifugation at 1500 rpm for 10 min. The supernatant was stored as virus stock at -20°C until used.

Virus assay

An attempt was made to use the most precise method for virus titration. Different cell cultures and media were used. The following is a description of the most efficient procedure which was employed. Approximately 1×10^6 MDC cells were seeded in each of the 60 x 15 mm plastic petri dishes and incubated at 37°C in a gas flow incubator with 3% humidified CO₂ in air. After 2 days, when complete monolayers were formed, 0.5 ml of a log dilution of virus in MEM was added to each culture. The virus was allowed to remain in contact with the cells for 1.5 hr at 37°C with frequent rotation at 15 min intervals. After the time of virus adsorption, the inoculum was removed and monolayers were washed with 2 ml of growth medium and overlaid with 5 ml of the following medium:

2x MEM (containing antibiotics)....	25 ml
2x HLA " "	25 ml
100x vitamins.....	1 ml
50x Eagle's amino acids.....	1 ml
Fetal calf serum.....	13 ml
2% special agar (Noble), sterilized by autoclaving.....	50 ml
7.5% sodium bicarbonate.....	3 ml

The plates were incubated in a gas flow incubator as described above. Ten days after incubation, 0.5 ml of 0.1% neutral red was added to each overlay. The plaques were counted the next day. The titre of stock virus was usually about 4×10^8 PFU/ml.

Electron microscopy

The monolayers of MDC cells were prepared in 3 oz. bottles (5×10^5 cells were seeded in each bottle and incubated at 37°C with 10 ml of MEM containing calf serum and antibiotics). The monolayers were inoculated with 2 ml of virus. The multiplicity of infection was 100 PFU/cell. The virus was allowed to adsorb for 1.5 hr at 37°C. After the adsorption period, the unattached virus was removed and the monolayers were washed with 3 ml of the prewarmed medium. The control (uninfected) and infected cells were incubated with 10 ml of fresh medium at 37°C. At varying time intervals, the medium was removed and the cell monolayers were washed once with 3 ml of chilled medium and fixed.

The following methods of fixation were applied:

1. Fixation in 4% commercial formaldehyde in 0.1 M phosphate buffer pH 7.2, containing 7.5% sucrose. (Holt and Hicks, 1961)
2. Fixation in 4% methanol-free formaldehyde (Pease, 1962; Robertson et al, 1963). This solution was prepared by dissolving 4 grams of paraformaldehyde powder in 100 ml of distilled water at 65°C. A few drops of 40% NaOH was added until the solution became clear. This fixative was used in phosphate buffer containing sucrose as mentioned above.
3. Fixation in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 (Leduc, 1967). The time of fixation was usually 1 to 2 hr, at 4°C.

The fixed cells were scraped off the culture bottles with a rubber policeman and centrifuged at 1500 rpm for 10 min. The pellet was washed with 0.1 M phosphate buffer pH 7.2 in four changes of buffer for a period of 16 to 24 hr. The pellet obtained by centrifugation was cut into small pieces with a razor blade, and dehydrated in the following solutions (Leduc, 1967), at 4°C:

1. 80% GMA (Polysciences, Rydal, Pennsylvania) in distilled water for 20 min.
2. 97% GMA in distilled water for 20 min.
3. Unprepolymerized embedding mixture which was prepared by mixing 7 parts of 97% GMA with 3 parts of butyl methacrylate containing 2%

Luperco (50% 2,4-dichlorobenzoyl peroxide with dibutyl phthalate) for 20 min.

4. Impregnation in prepolymer of embedding mixture, overnight. The preparation of prepolymer was as follows: a small amount of the GMA-butyl methacrylate-Luperco mixture was placed in an erlenmeyer flask so that a thin layer less than 1 cm in depth covered the bottom. The flask was heated over an open burner with rapid swirling of the contents until it started to boil. The flask was immediately cooled in an ice bath and agitated vigorously until the temperature of methacrylate dropped to that of the ice bath. The prepolymer had the viscosity of thick syrup at 0-3°C.

The cells were embedded in freshly prepared prepolymerized mixture using gelatin capsules held upright by a thin wire. Final polymerization was induced by ultraviolet radiation at 4°C with a lamp containing a fluorescent tube (Burton Manufacturing Co., Santa Monica, California). The distance of the top of the gelatin capsules from the tube was 1 cm. After 2 to 3 days, the blocks were hard enough to be sectioned.

Thin sections were cut, using a diamond knife (E. I. Dupont de Nemours and Company, Wilmington, Delaware) on a Porter-Blum MT-2 ultramicrotome (Ivan Sorvall, Inc., Norwalk, Conn.). The sections were picked up on 150 mesh

copper grids (E. F. Fullam, Inc., Schenectady, N. Y.), covered with carbon coated formvar films and stained with 3% uranyl acetate for 10 min and 0.4% lead citrate (Venable et al, 1965) for 5 min. The stained sections were examined with a Philips 200 electron microscope at 60 kV with single condenser illumination. Photographs were taken on Kodak 35 mm Fine Grain Positive film.

Shadowing

The grids, with sections, were mounted on a glass slide and shadowed with palladium in an EFFA Vacuum Evaporator (E. F. Fullam, Inc., Schenectady).

Digestion of thin sections with enzymes

An attempt was made to digest the enzyme-susceptible material completely with an optimum concentration of enzyme in a minimum period of time. The enzymes were obtained from Cal Biochem, Los Angeles, Calif. and were used as follows:

1. Deoxyribonuclease (Pancreatic, ^{38700 U/mg} B grade), at concentrations of 1%, 0.1%, 0.01% in veronal buffer containing 0.003 M MgSO_4 pH 7.0.
2. Ribonuclease (Pancreatic, ^{48 Kunitz U/mg} A grade), at concentrations of 1%, 0.1%, 0.01% in distilled water.
3. Pronase (B grade, ^{45 PUK U/mg}), at concentrations of 0.5%, 0.25%, 0.1% in HBSS, pH 7.2.
4. Trypsin (Difco Laboratories, ^{1:250}) at concentrations of 0.5%, 0.25%, 0.1% in PBS without calcium and magnesium.

Thin sections were picked up by a wire loop and floated on an enzyme bath and incubated at 37°C for 30, 60 and 90 minutes. After the incubation period, the thin sections were floated on ion exchange water (3 successive washes) and picked up on copper grids supported by a carbon coated formvar film. Treatment of thin sections with RNase and DNase was performed either directly or after digestion with proteolytic enzymes. In the latter case, the sections were first floated on proteolytic enzymes and incubated at 37°C, then washed 3 times with ion exchange water and transferred to nuclease solution for the further incubation.

Control sections were floated on the surface of distilled water and similarly incubated at 37°C.

The sections were examined in the electron microscope and the concentration of enzyme at which there was maximum digestion of susceptible material with a minimum destruction of cellular structures was chosen as the standard time and enzyme concentration for subsequent experiments.

Autoradiography

A. Incorporation of radioactive precursors

The MDC cell monolayers prepared in 3 oz. bottles were infected with ICL virus as described previously. The cells were labelled as follows:

1. Tritiated thymidine incorporation:

- a) Without chase: Control (uninfected) and infected cells were incubated in a medium

containing 50 μ c/ml tritiated thymidine, specific activity 20 c/mM (Schwarz Bioresearch, Inc., Orangeburg, New York), at 37°C for 1 hr. The cells were incubated at intervals of 11, 15, and 18 hr post inoculation. After the labelling time, the radioactive medium was removed and the cells washed 5 times with 5 ml of chilled MEM and then fixed in glutaraldehyde and embedded in GMA as described above.

- b) With chase: eleven hr after virus inoculation, control and infected cells were exposed to a medium containing 50 μ c/ml tritiated thymidine and incubated at 37°C for 1 hr. After the pulsing time, the radioactive medium was removed and the cells were washed 5 times with prewarmed MEM and chased in fresh medium (MEM without radioactivity) at 37°C for 7 hr. Then the cells were washed and fixed in glutaraldehyde.
- c) Before infection: the MDC cells grown in 3 oz. bottles were exposed to a medium containing 1 μ c/ml tritiated thymidine at 37°C. Twenty four hr later, the cells were washed with prewarmed MEM, infected with ICL virus, and incubated with fresh medium at 37°C. At 12, 16, and 20 hr after infection, the medium was

removed and the cells were fixed in glutaraldehyde. Uninfected cultures were similarly incubated with the radioactive precursor and fixed at the same times.

2. Incorporation of tritiated uridine:

Tritiated uridine (Schwarz Bioresearch, Inc.), specific activity 20 c/mM, diluted to 100 uc/ml in MEM containing 20 times excess of cold thymidine, was added to the cultures at 11, 15 and 18 hr after infection. The cultures were divided into 2 groups, one group was incubated at 37°C for 10 min and the other incubated for 30 min. After the incubation time, the cells were washed 5 times with chilled medium and subsequently fixed.

3. Incorporation of tritiated leucine:

The labelling of the cultures with tritiated leucine (Schwarz Bioresearch, Inc.), specific activity 40 uc/mM, diluted to 100 uc/ml in MEM was carried out as follows:

- a) Without chase: the infected cells were exposed to the radioactive medium at 11, 15, and 18 hr after inoculation for a pulse of 30 min at 37°C. The incorporation of radioactive leucine was stopped by washing the cultures with chilled medium and then fixing in glutaraldehyde.
- b) With chase: the cells were labelled at 12 hr

post infection for a pulse of 30 min at 37°C. After the labelling time, the cells were washed 5 times with prewarmed MEM, and then chased in fresh medium at 37°C for 6 hr, and subsequently washed and fixed.

- c) Before infection: the cells were exposed to tritiated leucine for 30 min at 37°C, then washed with prewarmed medium, infected with ICL virus and incubated with fresh medium at 37°C for 18 hr. After the time of incubation, the infected cells were washed with chilled MEM and fixed.

Control experiments were carried out with the same pulses on non-infected cells.

4. Estimation of tritiated uridine incorporation into RNA and DNA:

Eleven hr after virus inoculation, the infected cells were exposed to medium (MEM) containing 10 μ C/ml tritiated uridine at 37°C for 10 and 30 min. After the pulsing time, the cultures were chilled and washed 5 times with cold medium. The cells were scraped off the culture bottles with a rubber policeman and sedimented by centrifugation at 1500 rpm for 10 min. The pellet was resuspended in 1 ml of HBSS and the cells were disrupted by freezing and thawing (3 times). A 0.1 ml aliquot of this suspension was added to 5 ml of Bray's (1960) solution, and

assayed for radioactivity in a liquid scintillation counter (Nuclear Chicago).

The acid soluble compounds were removed by washing the disrupted cells with 2.5 ml of cold 10% TCA, twice. The nucleic acids were extracted by the procedure of Schmidt and Thannhauser (1945). An aliquot (0.1 ml) of each fraction was added to 5 ml of Bray's solution and assayed for radioactivity.

B. Preparation of the specimens and application of the emulsion

All the methods of fixation, dehydration, embedding and sectioning were carried out as described previously (see electron microscopy procedures). The application of the emulsion to the specimen is the most critical operation in the autoradiography technique. The layer of photographic emulsion should be thin, consisting of a monolayer of silver halide crystals which should be densely arranged and uniformly distributed. The following methods of layering the emulsion were employed:

1. Loop method (Caro and Van Tubergen, 1962).

The sections were picked up on 150 mesh gold grids supported with carbon coated formvar film and placed with the sections upward on the top of metal screw caps (2 cm in diameter) covered with parafilm. In the darkroom under safety illumination (Ruby safety bulb), 10 gm of Ilford L-4 emulsion (Ilford, Essex, England) was melted in 20 ml of distilled water at 65°C for 15 min (Caro and Van Tubergen, 1962). The

mixture was stirred thoroughly and the beaker was placed in ice for 3 minutes, then left at room temperature for 30 minutes. A wire loop, 3 - 4 cm in diameter, was dipped in the emulsion, and then slowly withdrawn so that a thin film was formed across the loop. The emulsion film was allowed to gel, then touched to the grids, dried for 10 min and kept in a light proof box for exposure.

This method did not produce a very homogeneous layer of emulsion and the development of individual grids was rather tedious.

2. Membrane method (Salpeter and Bachmann, 1965; Granboulan, 1965; Rogers, 1967).

Clean glass slides were marked with a diamond on one side of the slide at about 2 cm from the lower edge. The slides were dipped into a solution of 1% collodion (Parlodion in amyl acetate) and withdrawn slowly, dried at room temperature sheltered from dirt. The sections were picked up by a wire loop and laid onto the marks, but on the opposite side of the glass slide. The water was absorbed with a filter paper. The sections adhered to the membrane and the wire loop was then removed. In the darkroom the emulsion was melted at 45°C for 15 min and then diluted 1:6 in distilled water. The diluted emulsion was kept at room temperature for 1 hr with moderate agitation. The slides were dipped in the emulsion

and slowly withdrawn, then dried vertically and placed in storage boxes for exposure. After the exposure time and development, the collodion membrane, carrying sections, was stripped from the slide onto the surface of distilled water. The grids were placed gently over the sections and picked up from the surface of the water together with the collodion membrane.

Using this method, a packed monolayer of halide crystals over the sections could be obtained but it was difficult to float the membrane onto the surface of distilled water after the development in photographic solutions.

3. A thin film of collodion was formed on the surface of distilled water. The grids were placed on the surface of the membrane. The membrane with the grids was picked up on a glass slide and dried at room temperature. A relatively flat, smooth surface was formed on the surface of the grids covered with a thin layer of collodion. The sections were picked up by a wire loop and laid onto the grids mounted on the slide. Care was taken to avoid tearing the collodion membrane when the wire loop containing the sections was put on the grids. The water was absorbed with filter paper and the sections adhered to the membrane covering the grids. In the dark room, diluted emulsion (1:6) was prepared

and heated at 45°C for 15 min, then kept at room temperature for 1 hr with moderate agitation. The slides were coated by dipping, with slow vertical withdrawal from the emulsion. The slides were dried at room temperature for 15 min and kept in an exposure box.

This method was easy to handle, several grids (6) could be mounted on each slide and a relatively uniform monolayer of silver halide crystals could be obtained on the sections. (Fig. 1)

C. Exposure time

The slides (or grids) were kept in slide boxes and stored in a large light proof box at 4°C.

Since the exposure time depends on the dose of the label, the time of pulsing and the metabolism of the cell, it was difficult to predict the optimum time for exposure. The exposure time was usually determined by developing test slides at regular intervals. It varied from 2 weeks (tritiated thymidine labelled cells) to 1 month (tritiated uridine labelled cells).

D. Development

The grids were developed in D 19 (Kodak) at 18°C for 5 min (Granboulan, 1967), rinsed in distilled water and fixed in a solution of 25% sodium thiosulfate for 8 min. They were then washed in distilled water for 5 min and dried at room temperature.

E. Staining

Staining the sections before application of the

layer of emulsion (Salpeter and Bachmann, 1964) did not give satisfactory results, since stain was washed off in photographic solutions during the developmental process. The standard procedure was to stain the sections after the development of the emulsion. Without removing the gelatin layer, the sections were stained with 5% uranyl acetate in methanol (Venable et al, 1965) for 20 min and 0.4% lead citrate for 5 min.

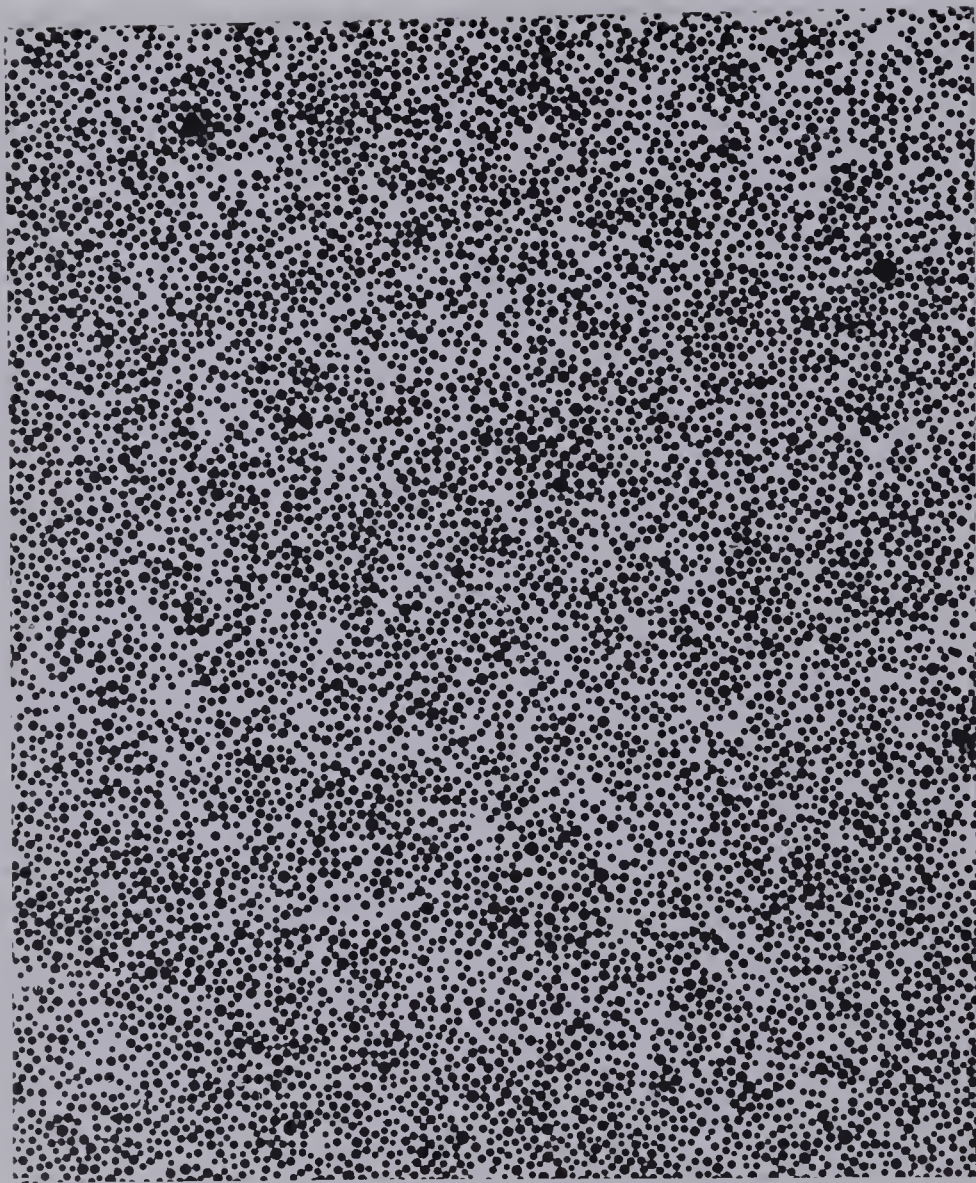


Fig. 1

Electronphotomicrograph of Ilford L-4 crystal in thin layer used for autoradiography.

The grids were mounted on a glass slide and covered with Ilford L-4 emulsion using the dipping method. They were exposed to light and examined in the electron microscope. A relatively packed monolayer of halide crystals formed over the grids, as shown in the photograph. X 7,000

RESULTS

Plaque assay of ICL virus

Several plaque assay methods were used for the titration of ICL virus. The method employed by Gaunt (1966) using DKL cell monolayers failed to give good plaques since the pH of the overlay medium became acidic after 3 days and the cell monolayers were not viable after 5 - 6 days.

Marusyk (1967) used another dog kidney cell line (MDC) for the assay of ICL virus. This method required several re-overlays of the monolayers before the plaques appeared and in some cases the plaques were not visible.

An attempt was made to establish a plaque assay method in which the re-overlay of monolayers could be avoided. Monolayers of MDC cells were prepared as described in Materials and Methods, then inoculated with 0.5 ml of virus suspension in MEM without calf serum. The virus was allowed to adsorb for 1.5 hr and then overlaid with 7 ml of overlay medium containing a mixture of MEM and HLA, 1% agar, and 10% fetal calf serum. The composition of the overlay medium and the detail of the procedures was described in Materials and Methods.

The plates were incubated at 37°C in an atmosphere with 3% CO₂ in air. Nine to ten days after incubation, neutral red was added to the monolayers and plaques (2 - 3 mm in diameter) appeared the day after. There was no increase in the number of plaques when the monolayers were

incubated for more than 2 weeks (Table I). The effect of calf serum prepared from the blood (obtained from the slaughter house) was investigated. An overlay medium containing 10% calf serum was used, and plates were incubated at 37°C. Nine days after incubation, no visible plaques appeared, but when the calf serum was replaced by fetal calf serum, clear plaques of a reasonable size were obtained. This could possibly be due to the presence of an inhibitor in the calf serum which inhibited the penetration of the virus into the cells.

TABLE I

THE EFFECT OF INCUBATION TIME ON THE NUMBER OF PLAQUES

Virus Dilution	# of Plaques after Incubation		
	10 days	12 days	15 days
10^{-6}	*	*	*
10^{-7}	45	48	44
10^{-8}	5	4	5
10^{-9}	0	0	0

* Too many to count

The monolayers of MDC cells in plastic petri dishes were inoculated with 0.5 ml of virus suspension in MEM without calf serum. The virus was allowed to adsorb for 1.5 hr at 37°C and then overlaid according to the procedure outlined in Materials and Methods. The plates were incubated in a gas flow incubator in an atmosphere of 3% CO₂ in air. The plaques were counted one day after the addition of neutral red.

When the plates were re-overlaid 6 days after incubation, no change either in the viable life span of the monolayers or the number of plaques was observed.

The use of 2x MEM either alone or mixed with an equal volume of ELA for the preparation of overlay medium did not produce plaques clear enough to be counted. The experiments in which primary dog kidney cells were used as monolayers for the assay method resulted in the cells not remaining viable for more than 5 - 6 days, and therefore plaques could not be obtained.

Electron microscopy

The preparation of thin sections for electron microscopy was described in Materials and Methods.

The preservation of cells fixed in formaldehyde was not satisfactory and the fine structure of cellular organelles was destroyed; for this reason, in all of the experiments, glutaraldehyde without osmium tetroxide was used for fixation of the cells.

The structural membranes (nuclear membrane, mitochondrial membrane, endoplasmic reticulum) of the cells fixed in glutaraldehyde and embedded in GMA appeared unstained as in negative contrast staining. This appearance is typical of the fixation of cells with glutaraldehyde without osmium tetroxide (Fig. 2).

1. Fine structure of uninfected MDC cells

The cells had a large nucleus, with one or two nucleoli visible in thin sections (Fig. 2). The chromatin was uni-

formly distributed throughout the nucleoplasm. The nucleolus was composed of fine granules embedded in a dense fibrillar network (nucleolonema) and light meshes filled with amorphous material. Small clusters of dark staining interchromatin scattered in the nucleolonema could be found. There were no inclusions visible either in the cytoplasm or in the nucleus of uninfected cells.

2. Appearance of early inclusions in infected cells

MDC cells were infected with ICL virus and 12 hr after infection, cells were fixed with glutaraldehyde and embedded in water soluble GMA. A large number of cells in thin sections showed irregular dark staining inclusions which were inside the nucleus, and did not have any structural resemblance to the nucleoli (Fig. 3). Since these inclusions were never observed in the nuclei of uninfected cells, they were considered to be virus specific inclusions.

3. Development of early inclusions into spheres

By 16 hr after infection, the majority of infected cells showed the presence of circular inclusions inside the nucleus (Fig. 4). These inclusions consisted of dark staining material surrounding a light staining central area. It is believed that these inclusions were formed by the development of the early inclusions into spherical structures which in thin section appeared as "rings" (Yamamoto, 1969). The number of these inclusions that

could be observed in a single nucleus varied from 1 to 4. They were found either separated or attached together and often appeared as a figure "8" (Fig. 5). No visible alteration could be observed in the cytoplasm up to this stage of infection. In some of the nuclei of infected cells, a few virus particles could be observed inside or outside the inclusions. This early appearance of virus particles was due to the advanced infectious process in some of the infected cells.

4. Further development of the ring form inclusions and the appearance of dark staining inclusions

At 18 hr after infection, the spheres (ring form inclusions) increased in size and the dark staining material of the outer part diffused into the central area. These merged to form a large mass in which the virus particles could be observed in groups surrounded by inclusion material (Fig. 6).

At this time, the appearance of dark staining inclusions which were circular in shape was observed in the cytoplasm and in the nucleus of infected cells (Fig. 7). As many as 3 inclusions could be observed in a single nucleus or cytoplasm, located usually near the nuclear membrane. In higher magnification, these dark staining inclusions appeared to have a dense granular structure with a regular border and could be readily defined from the rest of the cell contents (Fig. 8).

5. Appearance of light staining inclusions

At the time of the appearance of dark inclusions, a number of light staining inclusions with homogeneous structures could be observed in the nucleus (Fig. 7). These inclusions, which varied in size and shape, were surrounded by virus particles and usually appeared at the periphery of the nucleus. Up to 4 inclusions of this type could be observed in a single section of a nucleus, but they were never found in the cytoplasm.

After 18 hr, the cellular alteration was confined to the formation of virus crystals and the release of virus aggregate from the nucleus (Yamamoto, 1969).



Fig. 2

Electron photomicrograph of uninfected MDC cell fixed in glutaraldehyde and embedded in GMA.

The nuclear chromatin is evenly distributed. A few chromatin clumps are found along the nuclear membrane. The nucleolus is composed of fine granules embedded in nucleolonema with light meshes filled with amorphous material. Small clusters of dark staining interchromatin in the nucleolonema can be seen. The structural membranes are unstained as in negative contrasted material. X 10,000

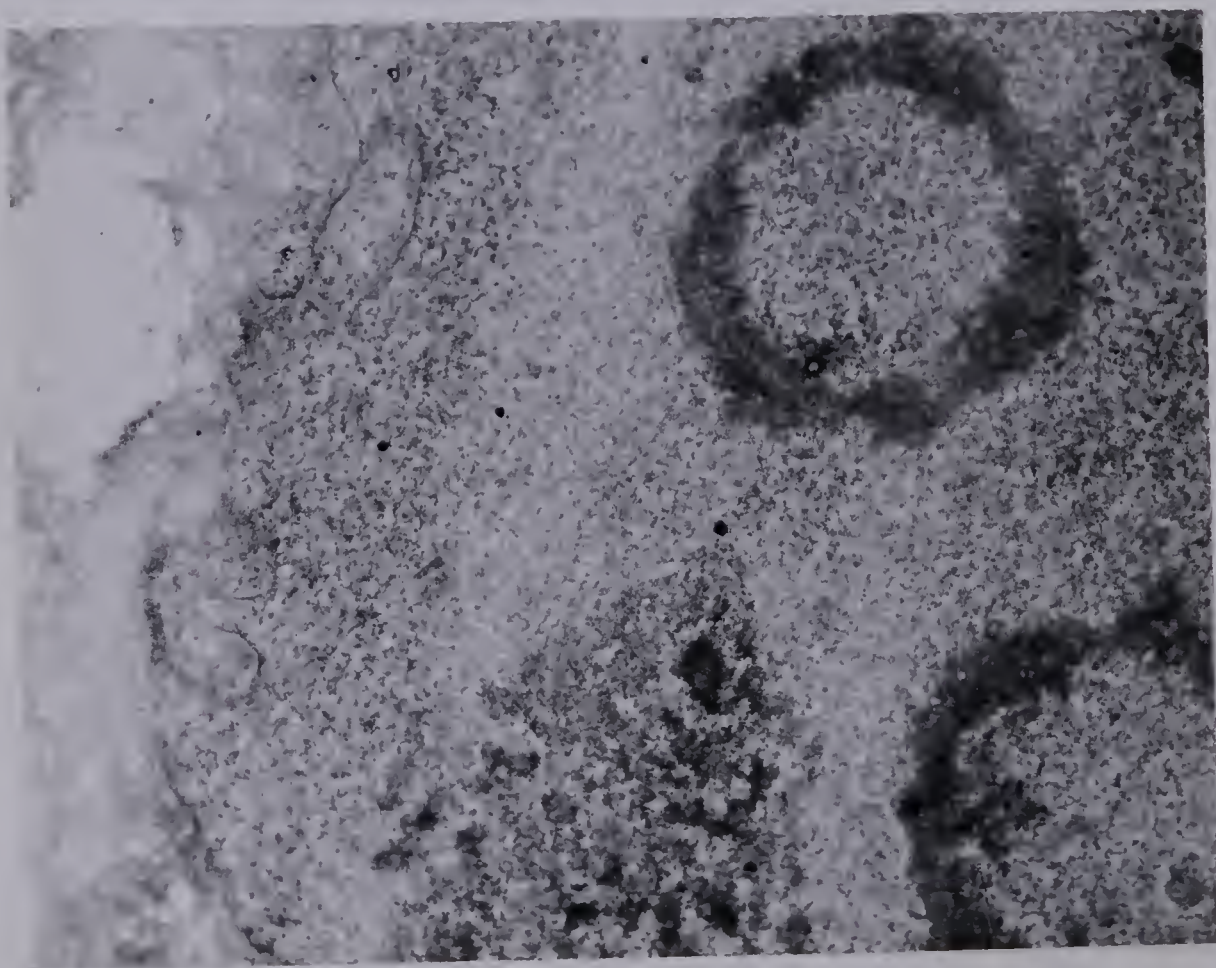
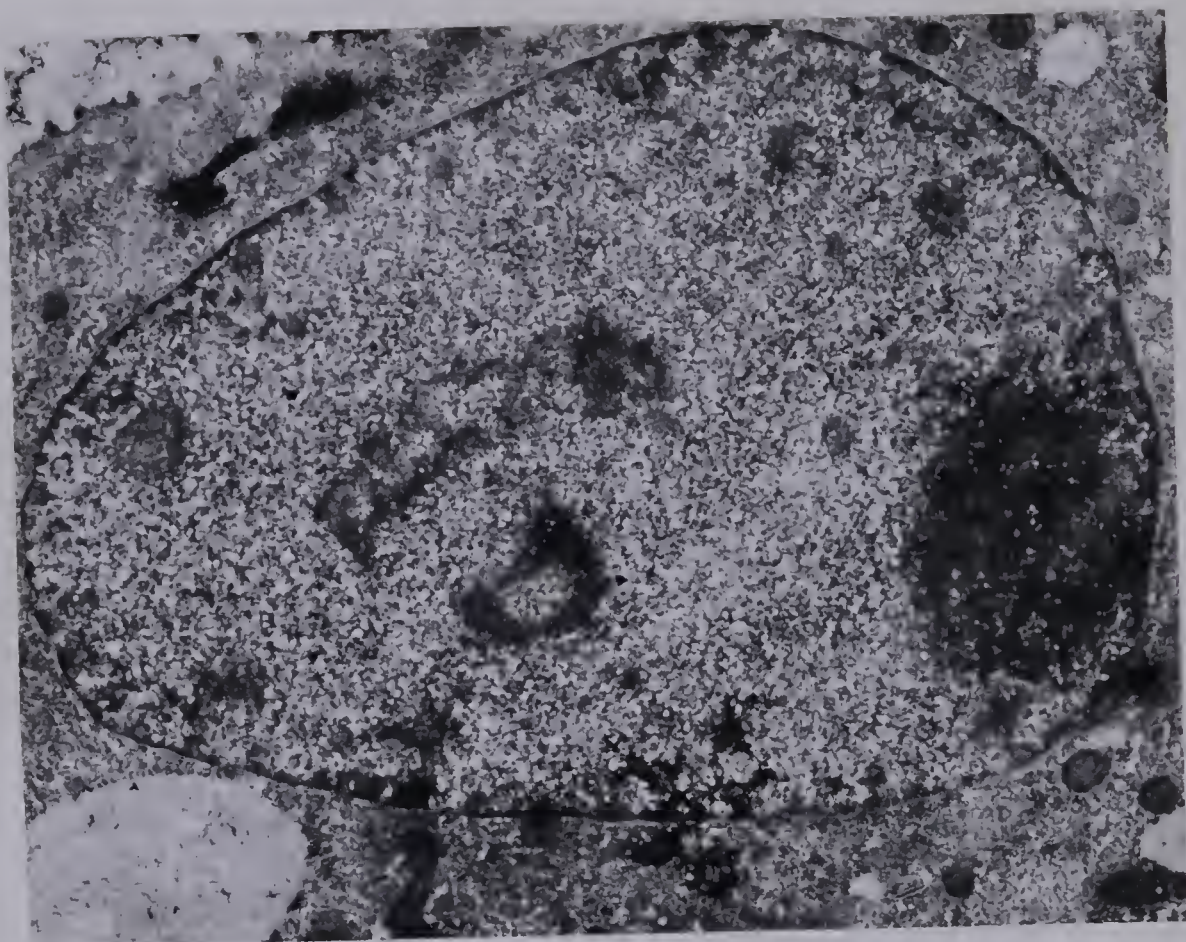


Fig. 3

MDC cell 12 hr after infection with ICL virus.
Early inclusions are seen inside the nucleus. The
nucleolus is observed near the nuclear membrane. X 11,500

Fig. 4

MDC cell 16 hr after infection.
The ring form inclusions with dark staining outer parts
surrounding light staining central areas are shown in
the nucleus. Part of the nucleolus also can be seen in
the left side of the photograph. X 16,000

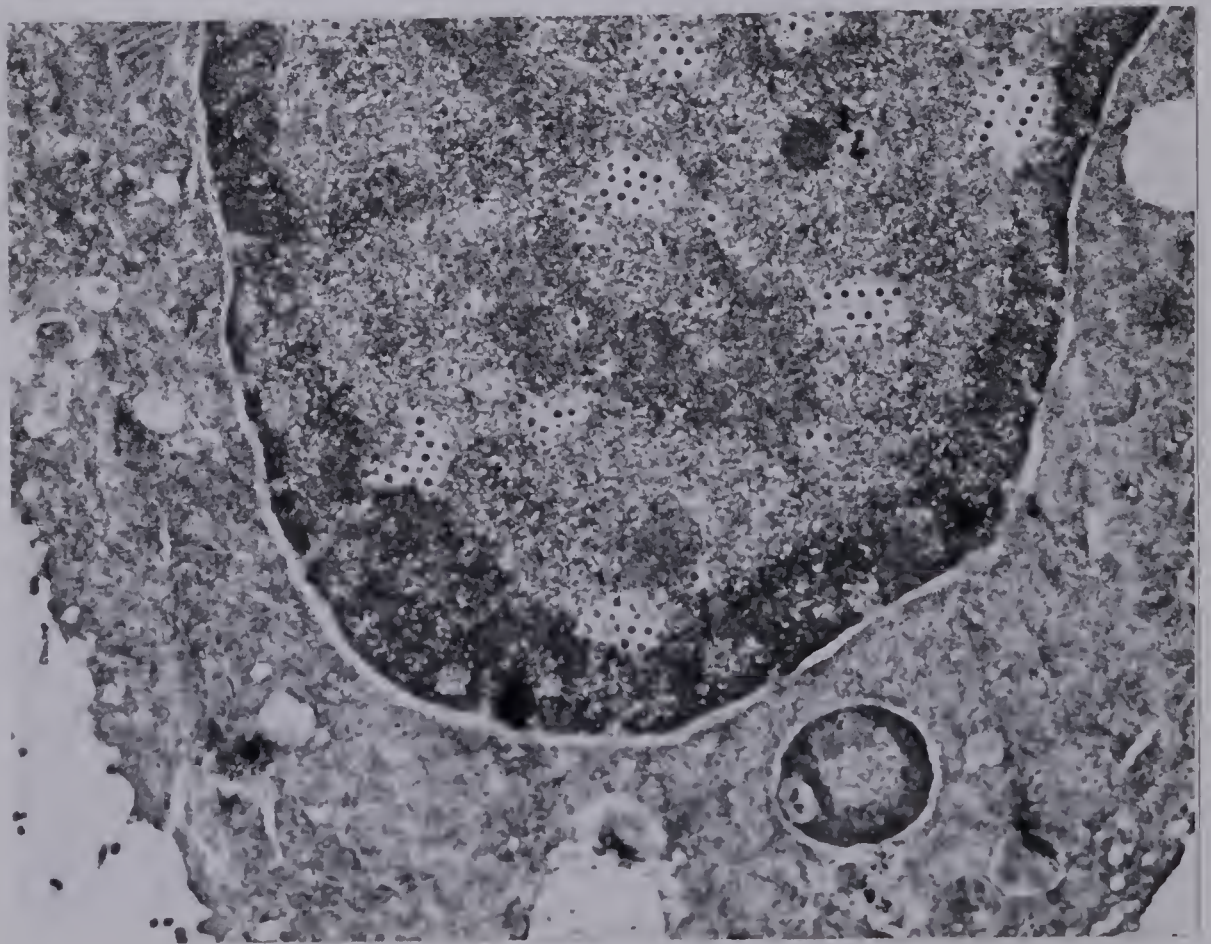
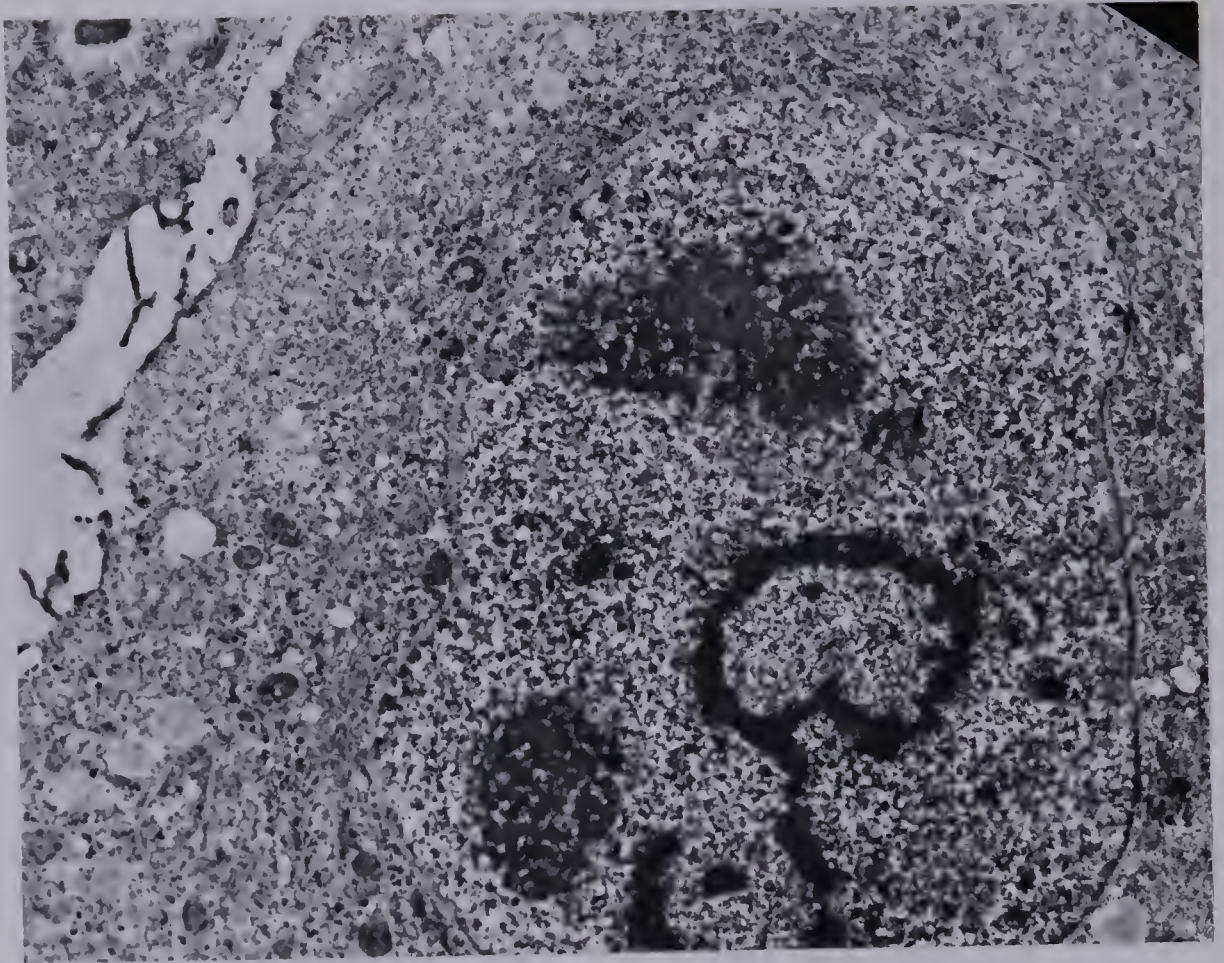


Fig. 5

The same infected cells as in Fig. 4.
The ring form inclusions are attached together and are seen as a figure "8". In addition, two nucleoli are observed inside the nucleus of the infected cell.
X 10,000

Fig. 6

MDC cell 18 hr after infection.
The ring form inclusions increased in size and the dark staining outer part diffused into the central area. These merged to form a single inclusion which can be seen as a large mass. Virus particles can be observed as groups surrounded by inclusion material. The peripheral dark staining material is the nuclear chromatin marginated toward the nuclear membrane.
X 13,000

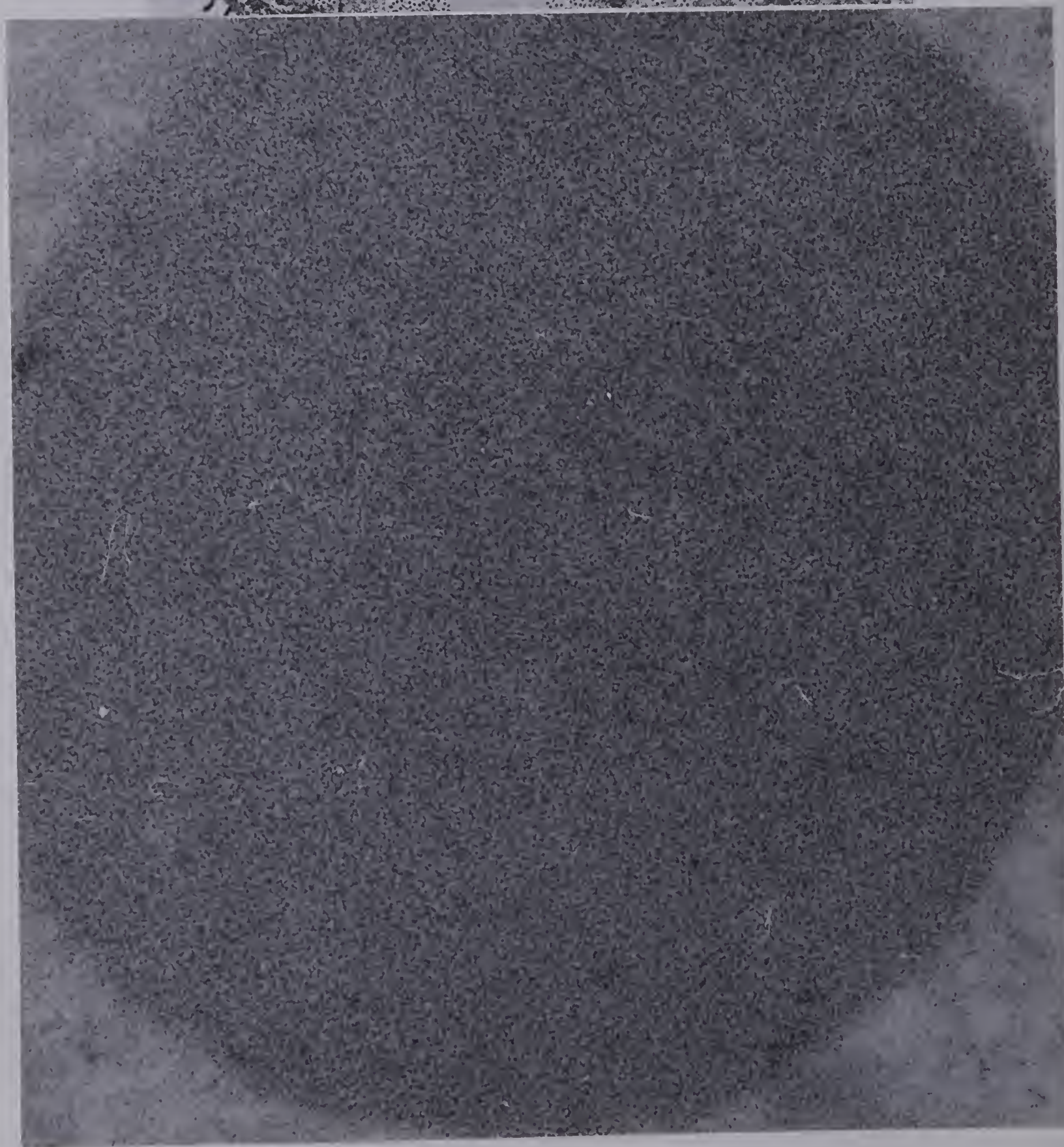
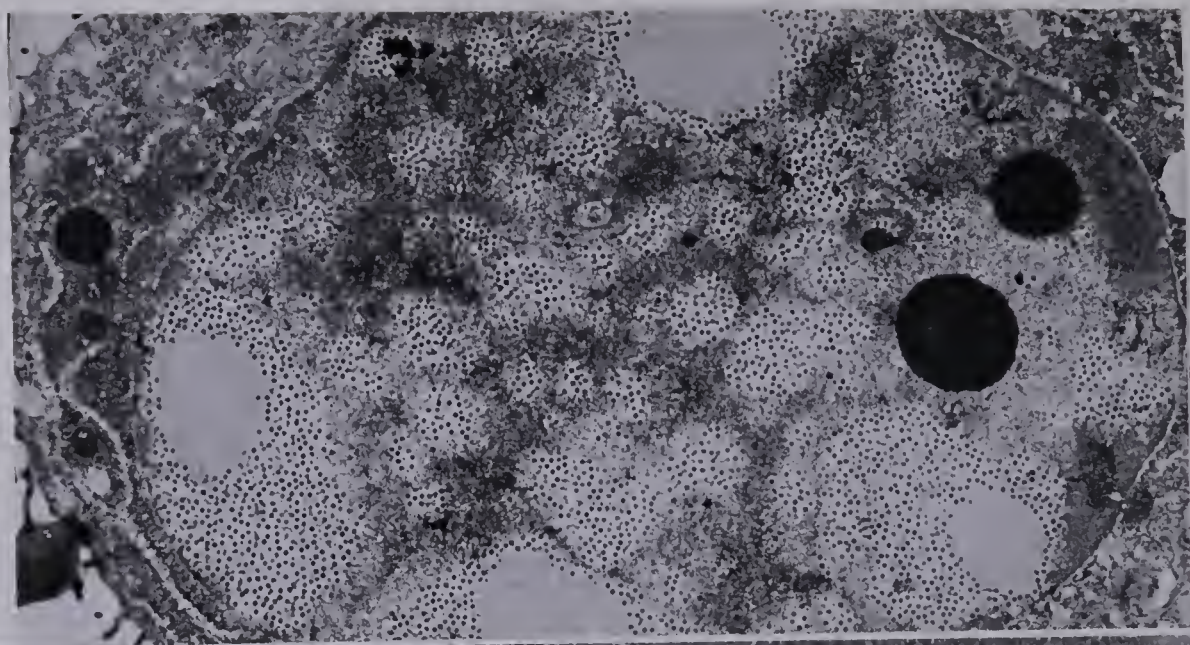


Fig. 7

MDC cell 20 hr after infection.

Dark staining inclusions are seen both in the nucleus and cytoplasm. Four light staining inclusions surrounded by virus particles are also observed inside the nucleus. The fibrogranular network inside the nucleus is the dark staining material of the ring form inclusions in which the virus particles are formed. X 8,000

Fig. 8

At high magnification, the dark staining inclusions appear to have a granular structure with a regular border. X 100,000

Cytochemical studies

Control (uninfected) and ICL virus infected cells were fixed in glutaraldehyde at intervals of 12, 16, and 20 hr after inoculation. The fixed cells were embedded in water miscible GMA and sectioned as explained in Materials and Methods. Before treating the sections with enzymes, it was decided to determine the optimum concentration of enzyme solutions for the digestion of susceptible material without the complete destruction of cellular organelles.

1. Digestion with proteolytic enzymes

The sections were floated on solutions of pronase at concentrations of 0.5%, 0.25% and 0.1% in HBSS pH 7.2, and of trypsin at concentrations of 0.5%, 0.25% and 0.1% in PBS without Ca^{++} and Mg^{++} pH 7, and incubated at 37°C. At intervals of 30, 60, and 90 min, sections were removed, washed with distilled water and after staining with uranyl acetate and lead citrate, examined in the electron microscope. It was found that the 30 min digestion of thin sections with either pronase or trypsin, at a concentration of 0.25% gave adequate digestion of the protein containing inclusions.

2. Treatment of thin sections with RNase and DNase

Similar procedures (as mentioned above) were carried out for the digestion of sections with nucleases. Solutions of RNase and DNase at concen-

trations of 1%, 0.1% and 0.01% were prepared according to the Materials and Methods. The sections were subjected to digestion at 37°C either directly or after treatment with proteolytic enzymes, for 30 min, 1 hr, 1.5 hr and 2 hr. In the case of direct treatment with the nucleases, no visible effect was apparent on the components of the nucleus or cytoplasm even with much longer digestion times (4 hr). However, incubation of the sections in 0.1% nucleases for 1 hr following treatment with proteolytic enzymes resulted in the disappearance of nucleic acid containing components. Simultaneously, consecutive sections were incubated in distilled water as controls in each experiment for non-specific extraction.

The appearance of uninfected and early infected cells after enzyme digestion

Thin sections prepared from the uninfected and 12 hr infected cells were treated either with 0.25% pronase or with 0.25% trypsin, for 30 min. Digestion of the uninfected cells with proteolytic enzymes resulted in the general loss of contrast, particularly in the nucleoplasm (Fig. 9). Some of the nucleolar material was removed, leaving the interchromatin clusters scattered in a granular stroma.

The early inclusions in infected cells were largely digested, but not completely removed and their stain intensity was much less compared to that of the nuclear

chromatin (Fig. 10). The intensely staining material margined toward the nuclear membrane was much less affected by the enzyme than were the early inclusions.

Incubation of the thin sections with 0.1% DNase for 2 hr did not show any effect on the staining character of the nuclear component. However, when DNase treatment was preceded by proteolytic enzyme digestion, the inclusions were almost completely removed, indicating that they were composed of protein and DNA (Fig. 11). The dark staining material along the nuclear membrane also disappeared. This condensed material, sensitive to DNase, was probably margined nuclear chromatin.

RNase at a concentration of 0.1% did not appear to attack the inclusions either before or after proteolytic enzyme digestion, except to generally reduce the background stain and hence increase the contrast of the organelles (Fig. 12).

The results obtained from the digestion of the ring form inclusions with proteolytic enzymes and nucleases were similar to those found for the early inclusions (Fig. 13, 14, and 15). It seems that these inclusions (rings) were formed by the development of the early inclusions (as observed by Yamamoto, (1969) in primary dog kidney infected cells) and had a similar texture.

Digestion of dark and light staining inclusions

Twenty hr infected cells in sections incubated in either 0.25% pronase or 0.25% trypsin, exhibited the

complete removal of dark, and light, staining inclusions (Fig. 16). The irregular clear areas surrounded by virus particles correspond to those areas occupied by the light staining inclusions and the circular clear areas represent the digested inclusions which were observed as dark staining circles in the control sections (Fig. 17). These inclusions were not affected by DNase before treatment with proteolytic enzymes (Fig. 18).

In order to determine the nature of the clear areas and whether they were the result of removal of proteins by proteolytic enzymes or due to reduction of stain intensity, the thin sections were shadowed with paladium before and after proteolytic enzyme digestion. A shadow was formed at the boundary of the clear areas in the digested sections (Fig. 19), indicating the complete removal of inclusions (compare Fig. 19 and 20 to note the disappearance of material from the circular inclusions). However, incubation of the thin sections in 0.1% RNase at 37°C for 2 - 4 hr either before or after protease digestion did not have any visible effect on the ultrastructural appearance of inclusions (Fig. 24 and 25).

Digestion of virus particles

In sections digested with proteolytic enzymes, the virus coat proteins were removed leaving the virus DNA core, which appeared as dark spots located in the centre of light circles (Fig. 21). Following this treatment (with proteolytic enzymes), digestion with 0.1% DNase for a

short time (30 min) resulted in the disappearance of viral DNA core (Fig. 22).

Treatment of the thin sections with proteolytic enzymes for a short time (15 min) followed by DNase digestion (for 1 hr) resulted in the removal of viral DNA core leaving viral coat proteins (Fig. 23).

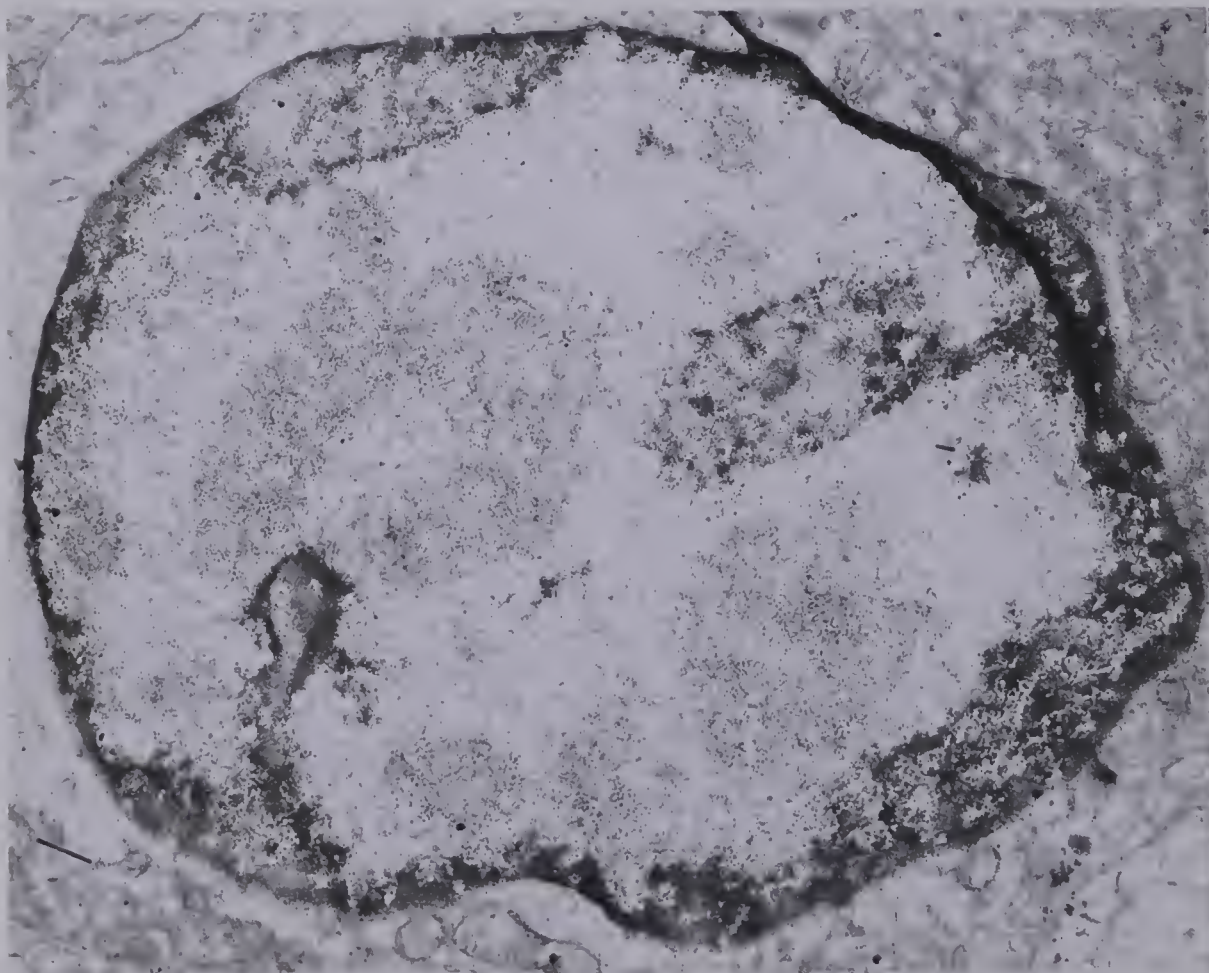
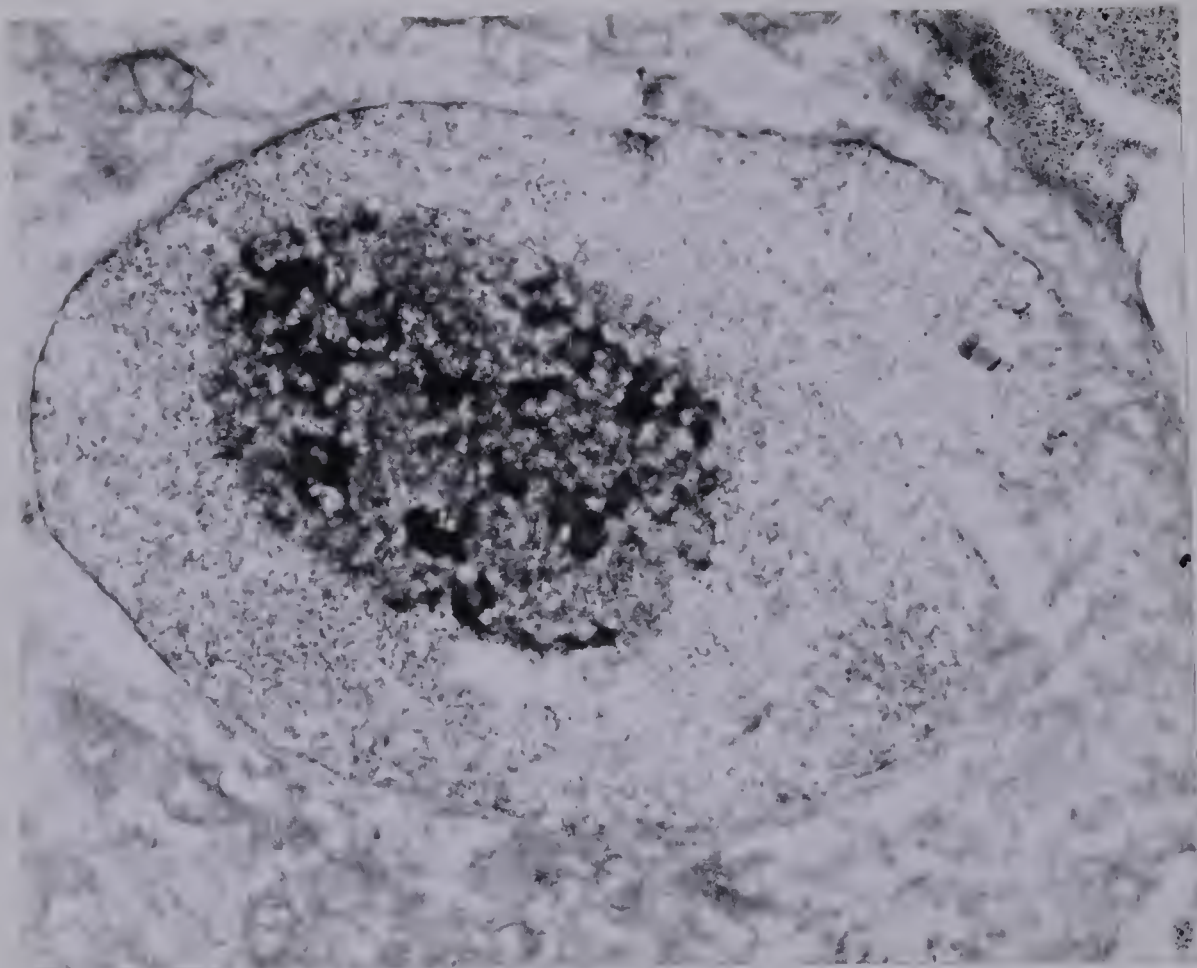


Fig. 9

Uninfected MDC cell.

Thin section was floated on a 0.25% pronase solution for 30 min. A general loss of contrast in nucleoplasm is observed. Part of the nucleolar material is removed and the interchromatin are seen scattered in a granular stroma. X 14,000

Fig. 10

MDC cell 12 hr after infection digested with pronase (0.25%) for 30 min.

The early inclusions are partially digested and have very low stain intensity. The marginated material toward the nuclear membrane seems to be less affected by the enzyme than the inclusion. A small part of the nucleolus (at top right) and invagination of the nuclear membrane (at bottom) can be seen. X 11,500

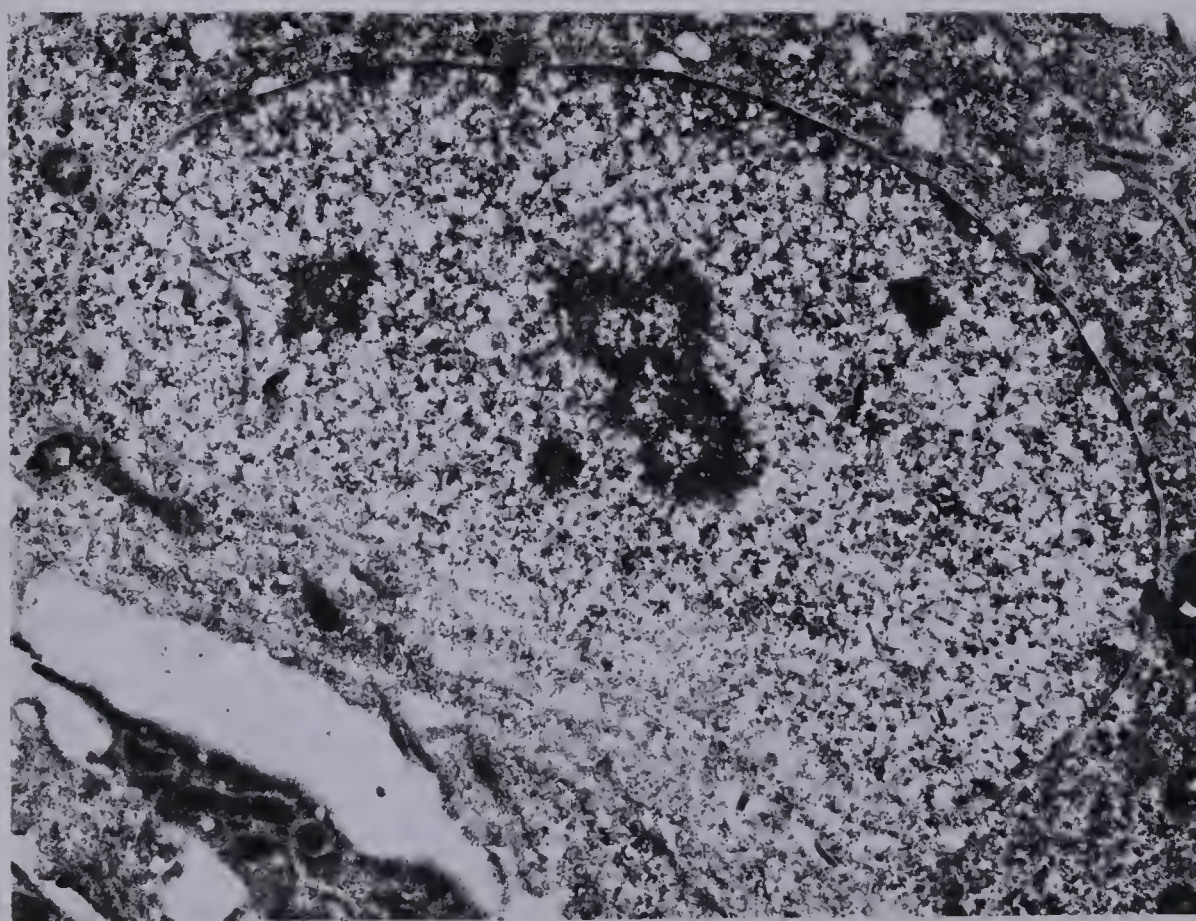


Fig. 11

MDC cell 12 hr after infection.

Thin section was treated with trypsin (0.25%) for 30 min, then digested with DNase (0.1%) for 1 hr. The early inclusions are removed and the marginated material toward the nuclear membrane is digested. X 8,000

Fig. 12

MDC cell 12 hr after infection.

The section was floated on RNase solution (0.1%) for 2 hr at 37°C. No detectable change can be seen in the appearance of early inclusions except the increase of the contrast of these bodies. X 11,500

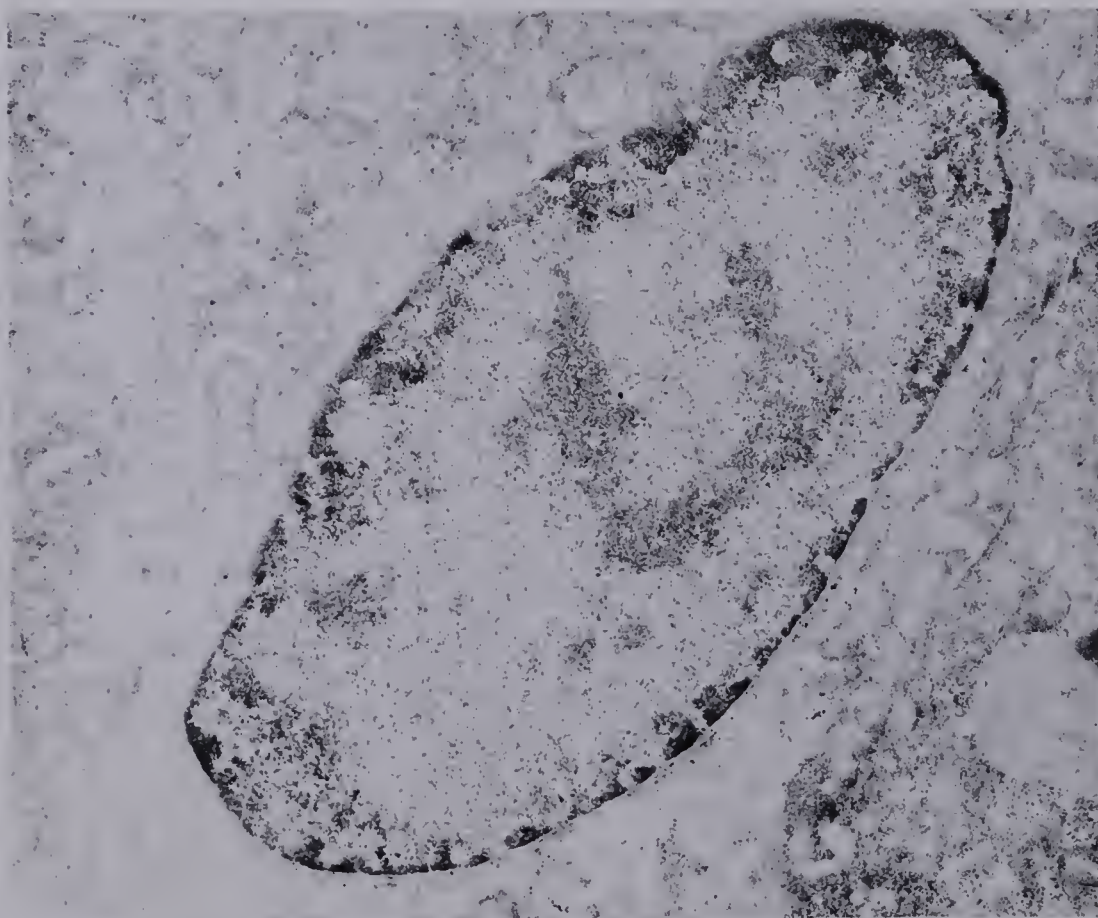
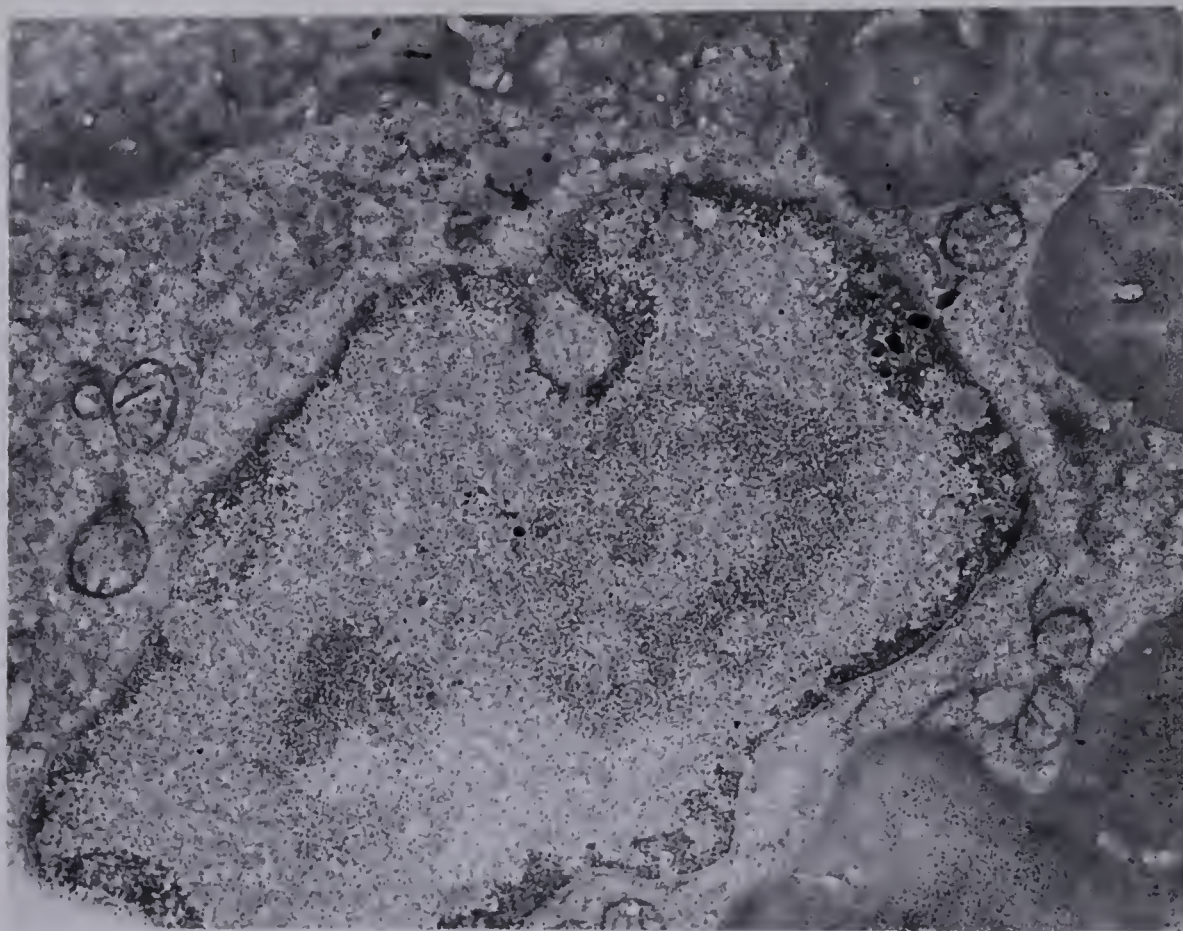


Fig. 13

MDC cell 16 hr after infection.
The section was treated with trypsin (0.25%) for 30 min.
The ring form inclusion is partially digested and has a
lower stain intensity than the marginated chromatin along
the nuclear membrane. X 11,000

Fig. 14

MDC cell 16 hr after infection.
The section was treated with trypsin for 30 min, then
digested with 0.1% RNase for 1 hr. No detectable changes
in the appearance of the ring form inclusion can be
observed. X 11,000

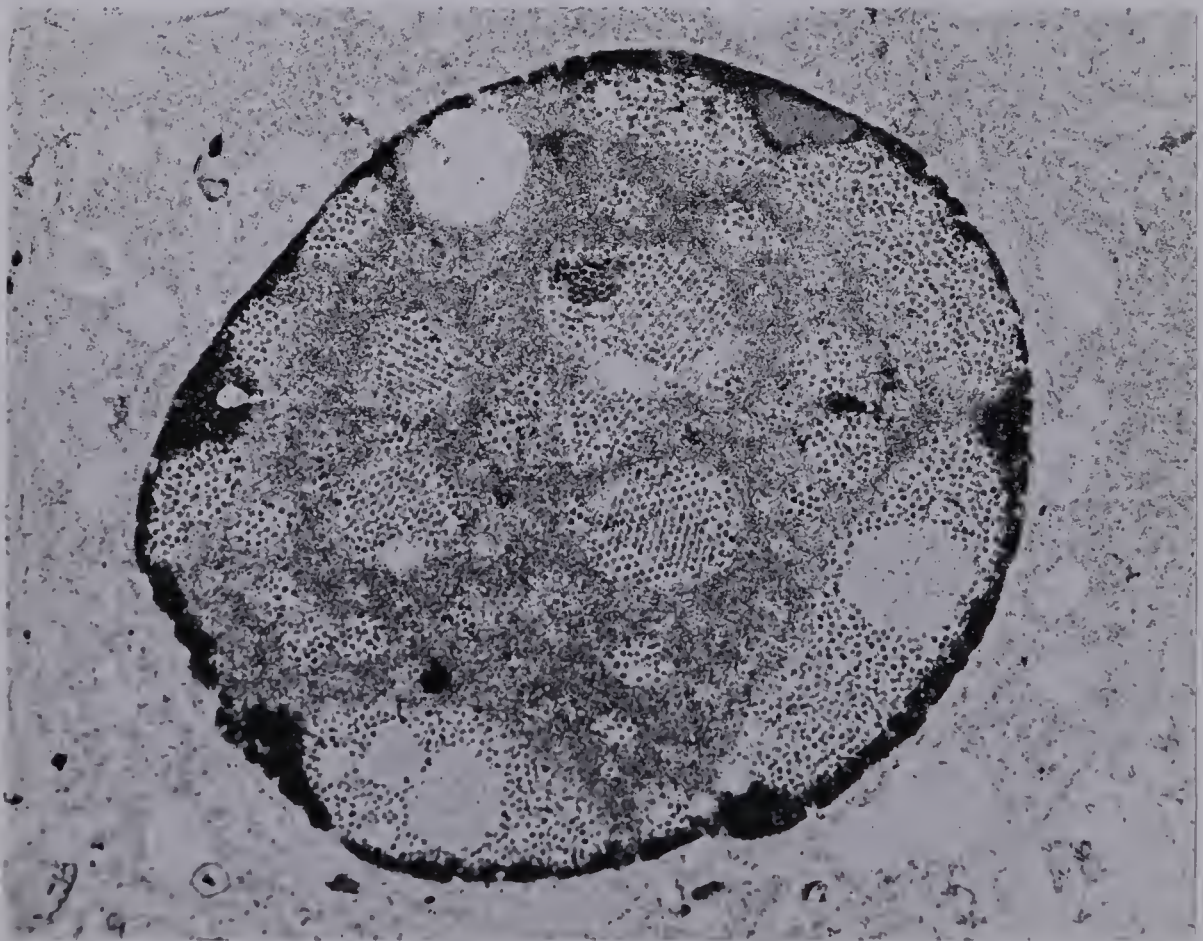
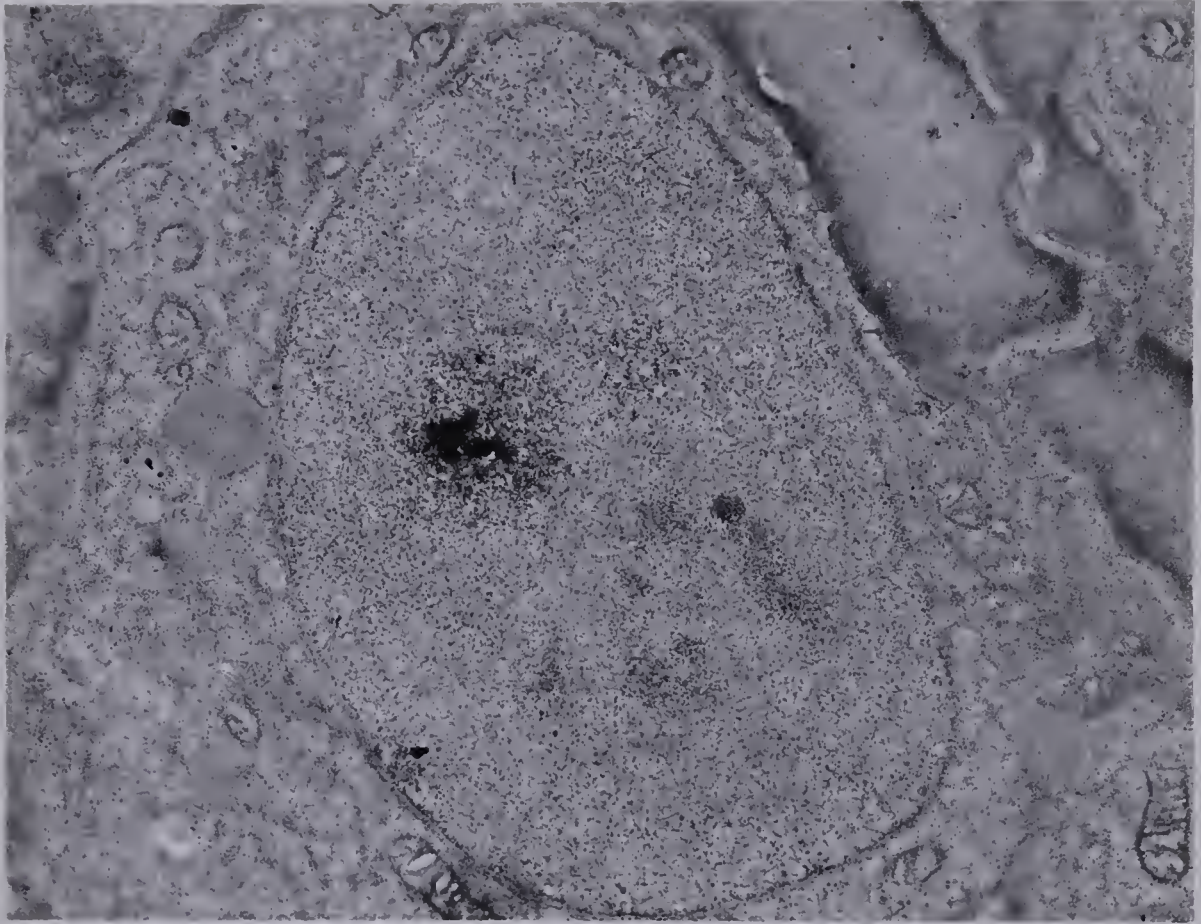


Fig. 15

MDC cell 16 hr after infection.

Thin section was floated on a solution of 0.25% trypsin for 30 min, then digested with 0.1% DNase for 1 hr. The ring form inclusion is digested. In the centre, the partially digested nucleoli are seen. X 11,000

Fig. 16

MDC cell 20 hr after infection.

The section was digested with 0.25% pronase for 30 min. Dark staining inclusion (at top) is completely removed. Light staining inclusions surrounded by virus particles are also digested. The fibrogranular central network is partially digested (compare its stain intensity with marginated chromatin). X 5,500

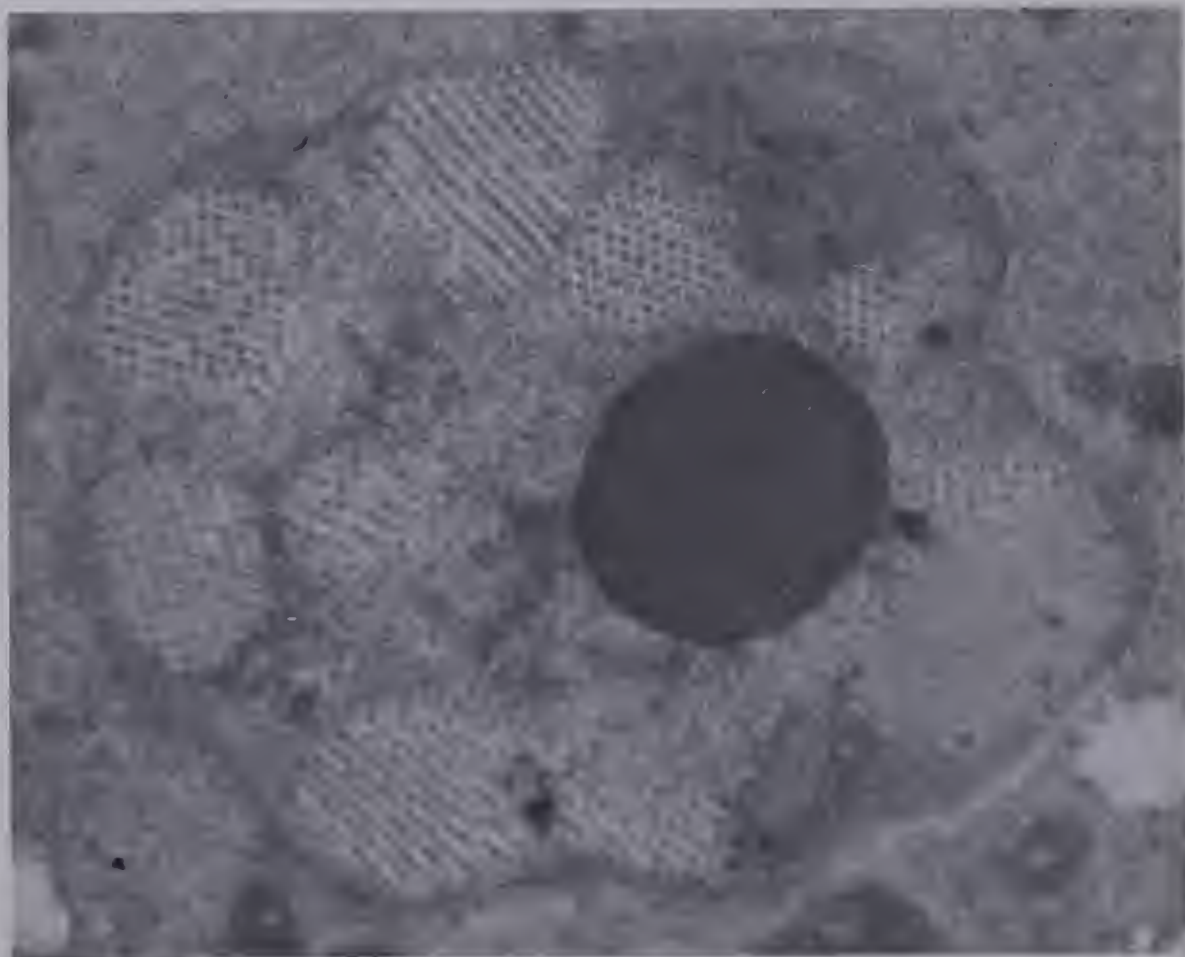
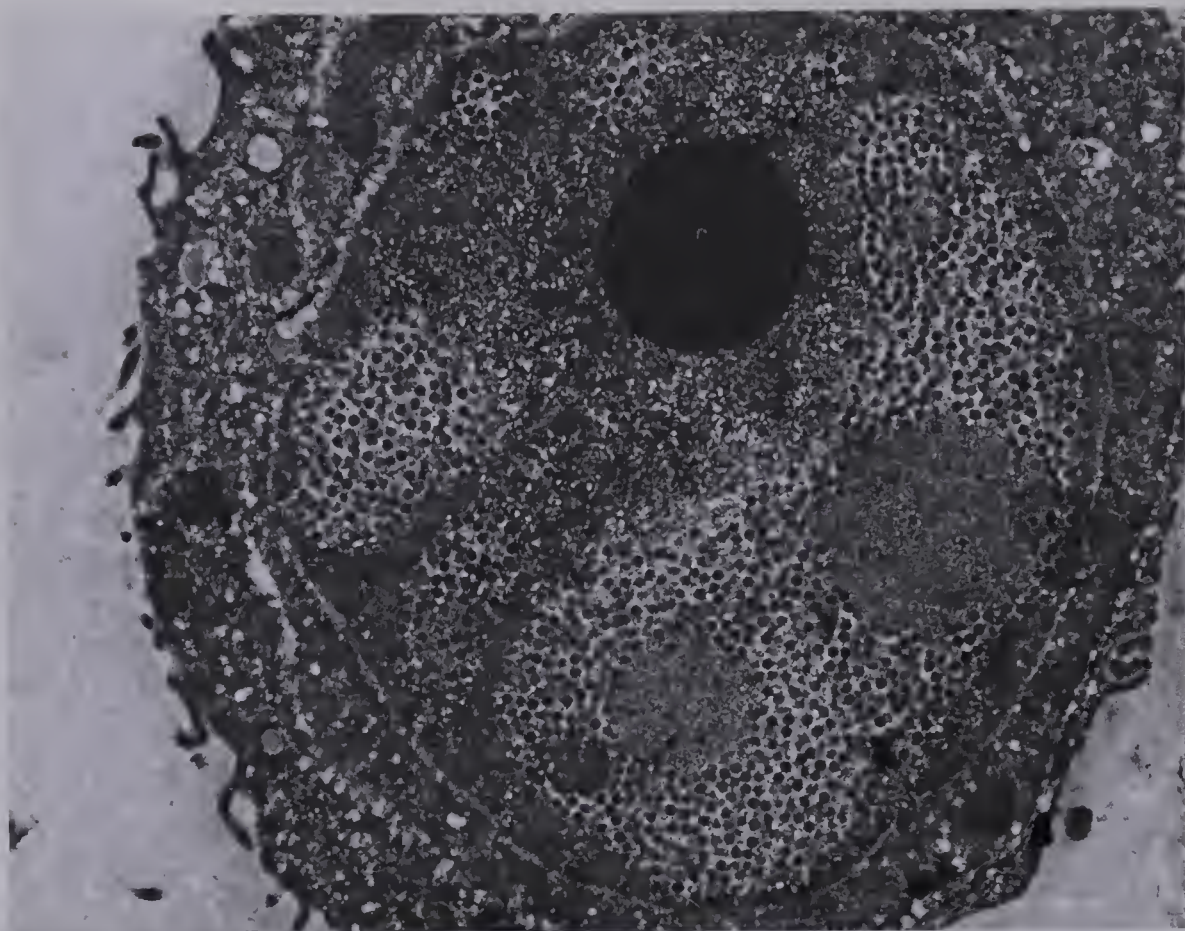


Fig. 17

A cell in the same stage of infection as that in Fig. 16. The section was floated on distilled water at 37°C for 30 min. No change in the appearance of dark and light staining inclusions can be observed. X 7,000

Fig. 18

A cell in the same stage of infection as in Fig. 16. The section was treated with 0.1% DNase for 1 hr. Dark and light staining inclusions are unaffected. X 11,500

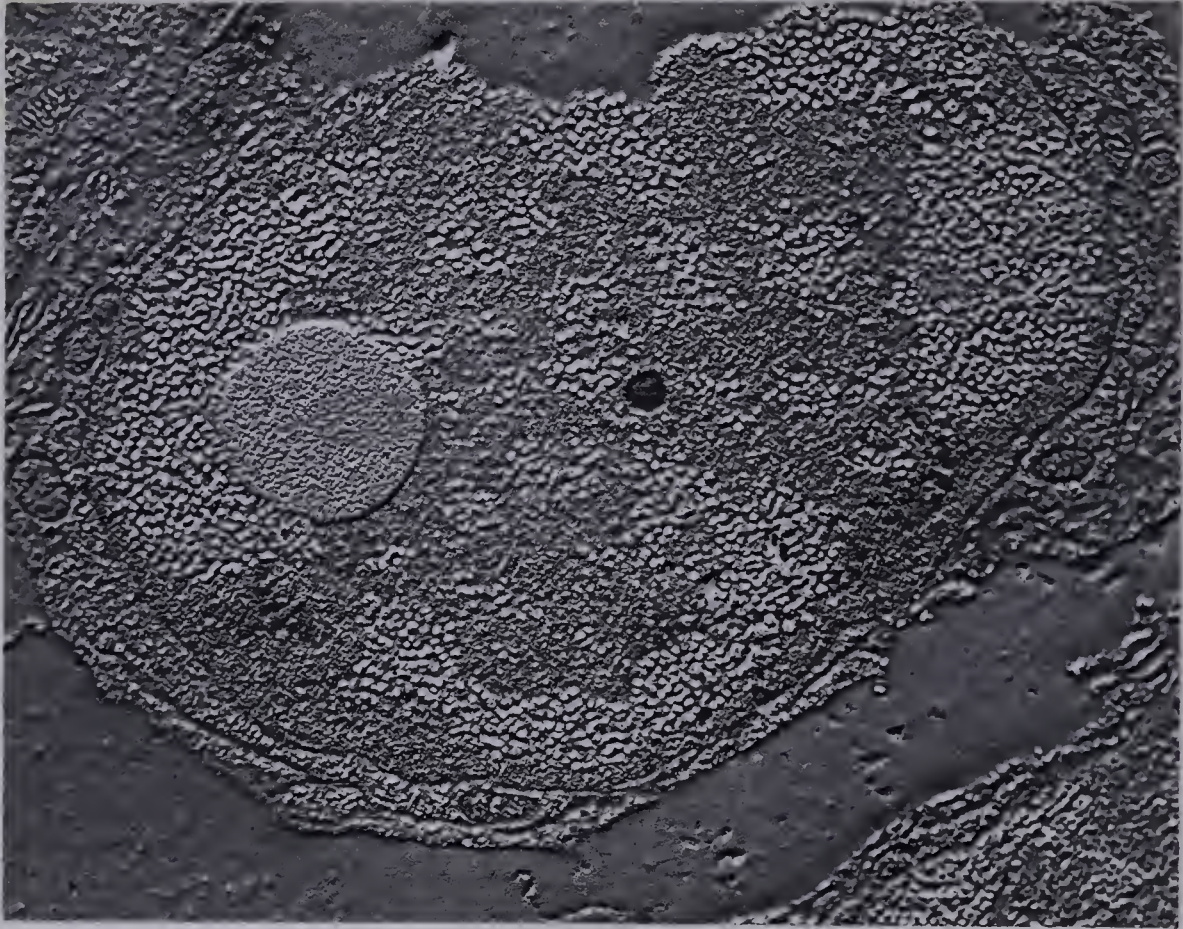


Fig. 19

MDC cell 20 hr after infection digested with trypsin and shadowed with paladium.
The shadow is formed at the boundary of the digested dark staining inclusion. X 10,000

Fig. 20

Control (undigested) MDC cell 20 hr after infection and shadowed as in Fig. 19.
No shadow is formed at the boundary of the inclusions.
X 7,000

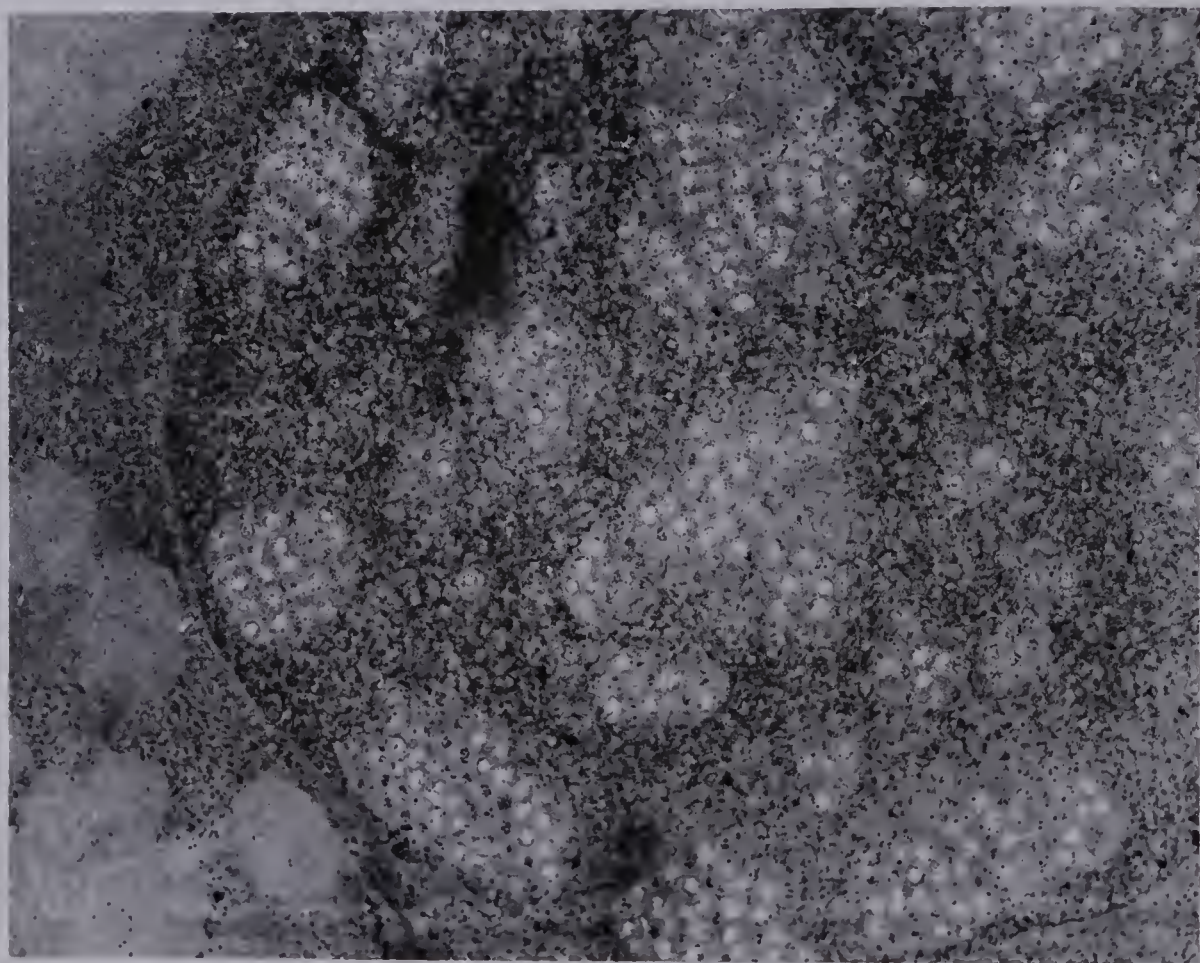
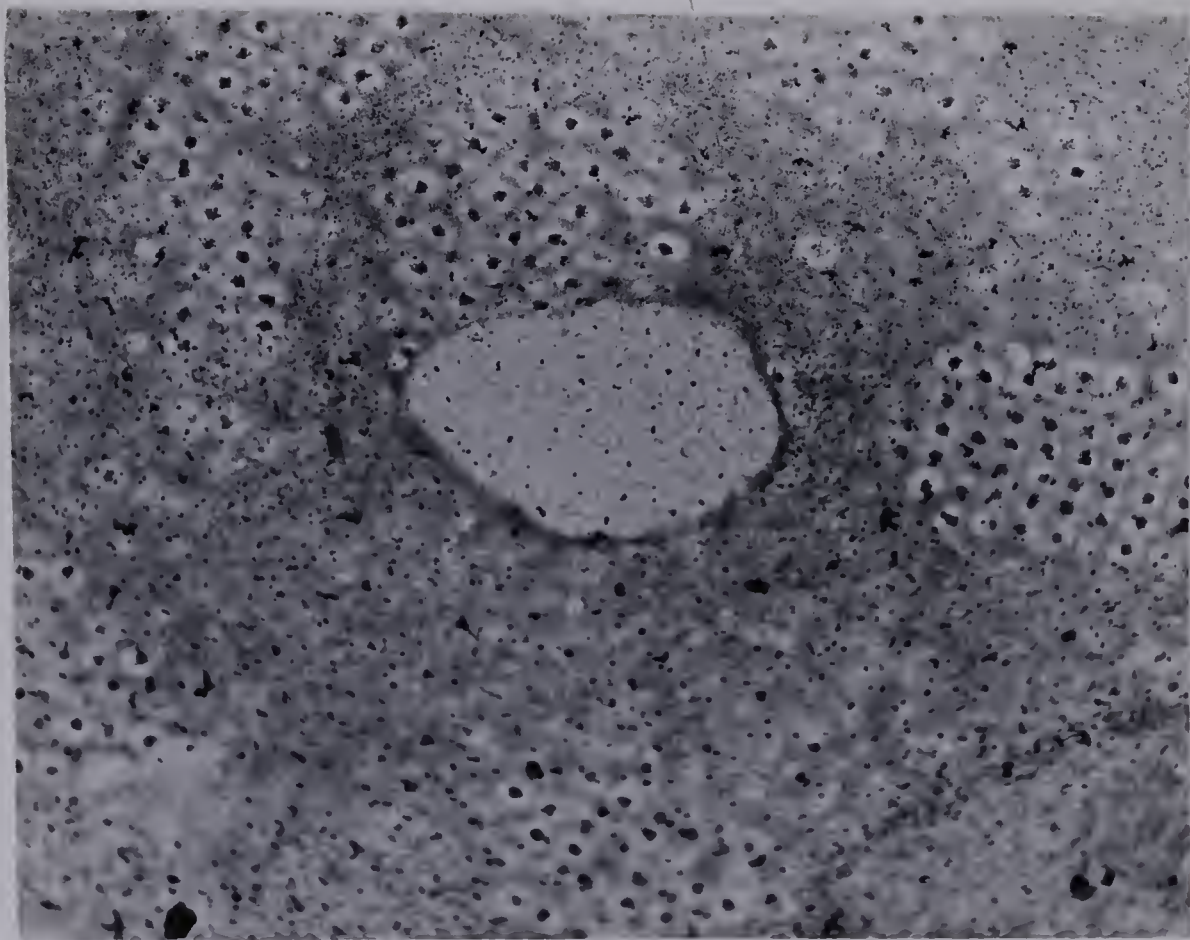


Fig. 21

ICL virus in MDC cell.

The section was treated with trypsin for 30 min. The virus coat proteins are removed, leaving the virus DNA core (dark spots in the centre of clear circles). In the centre of the picture, invagination of nuclear membrane.
X 23,000

Fig. 22

ICL virus in MDC cell.

The section was treated with trypsin then digested with DNase for 30 min. The virus particles are removed (clear circles). X 17,000

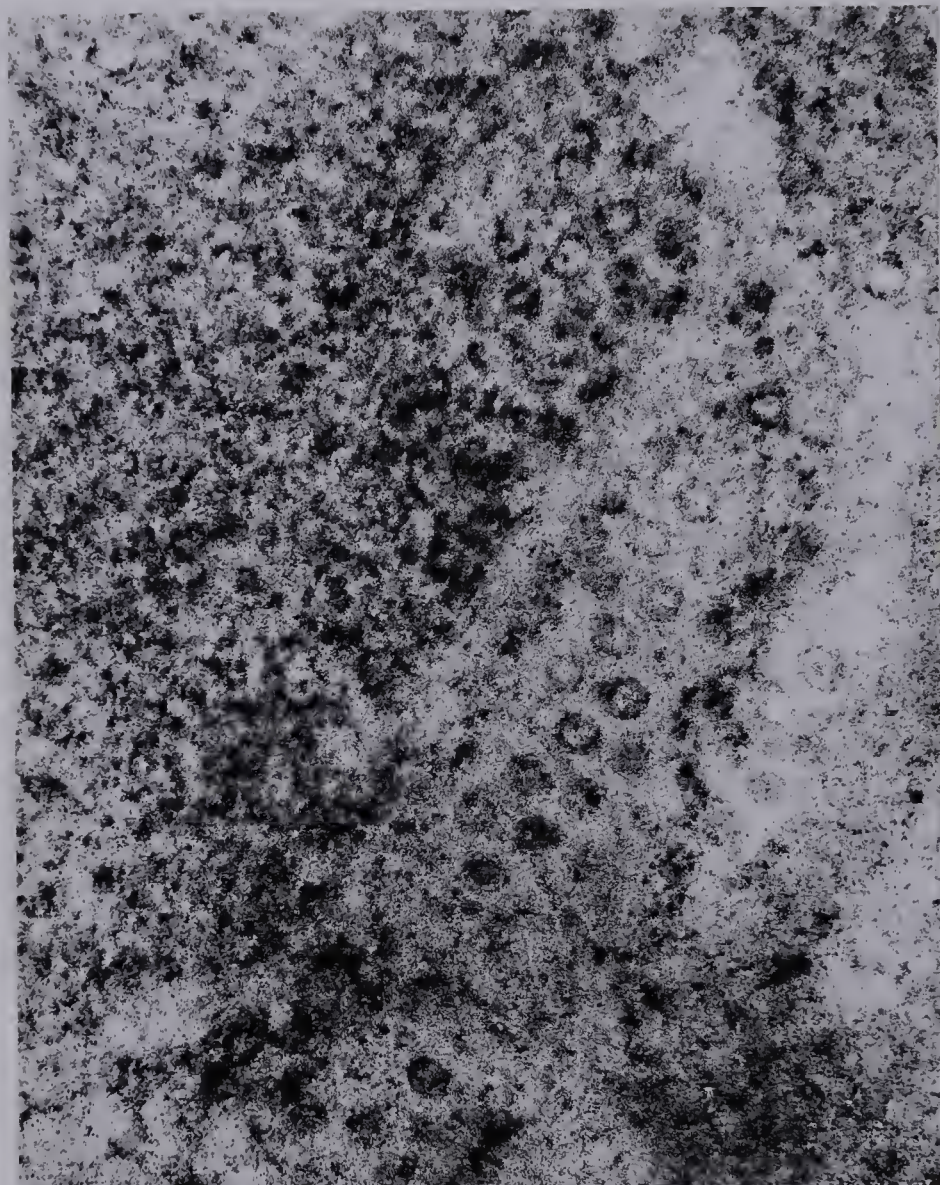


Fig. 23

ICL virus in MDC cell.

The section was treated with trypsin for 15 min then digested with DNase for 30 min. The virus DNA core is digested but the viral coat proteins are not completely removed. X 39,000

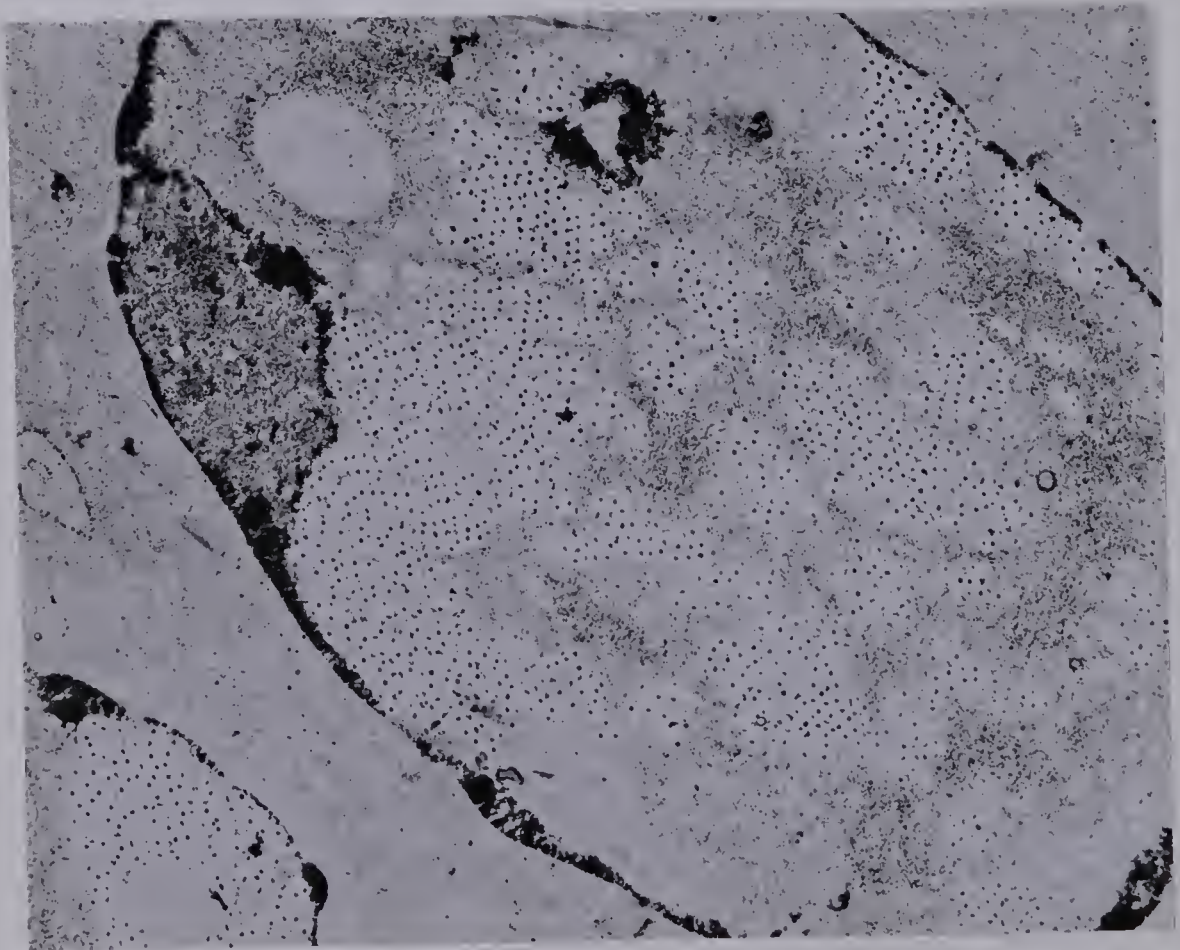
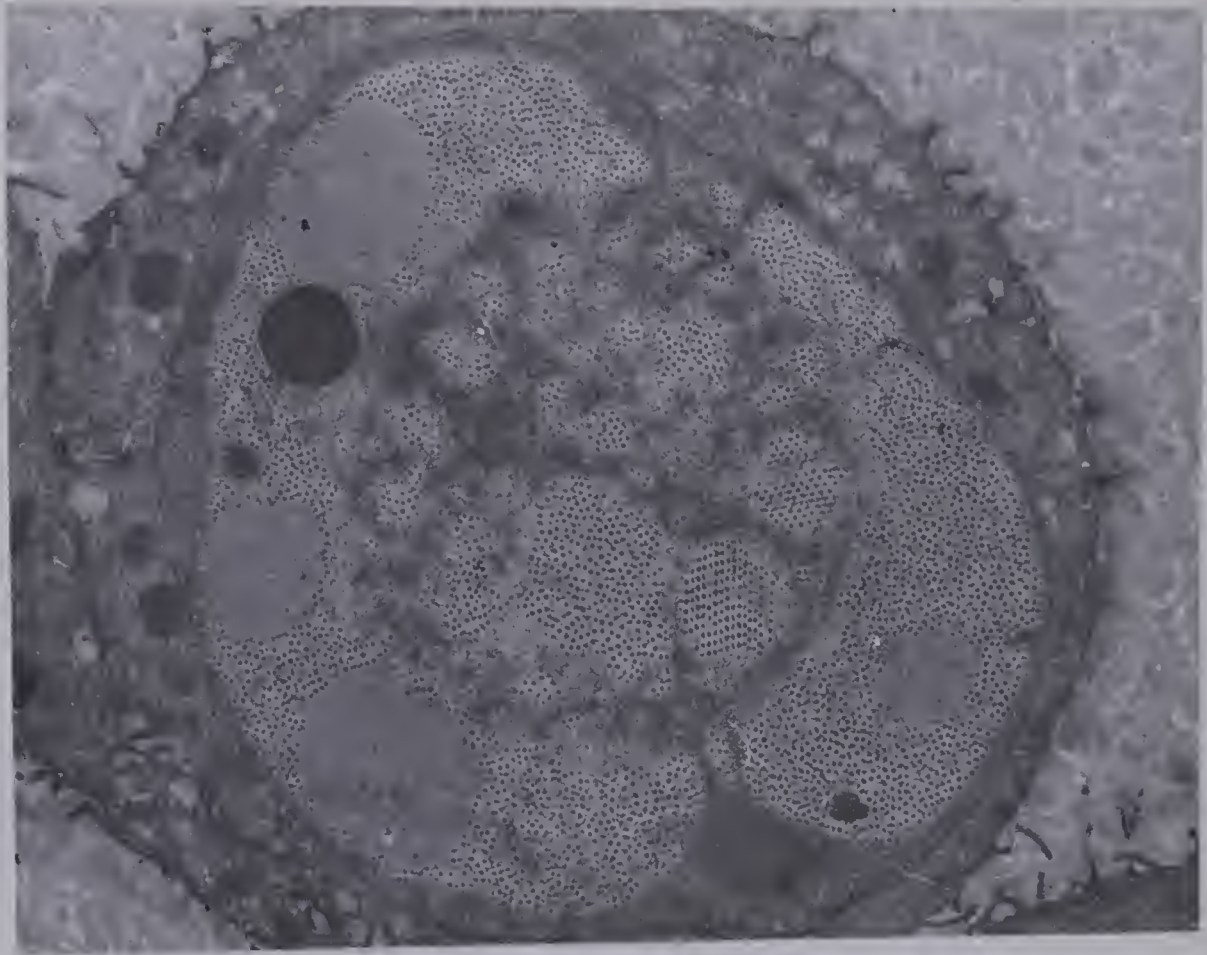


Fig. 24

MDC cell 20 hr after infection.

The section was floated on a solution of 0.1% RNase for 2 hr. None of the inclusions appear to be attacked by the enzyme. X 8,000

Fig. 25

20 hr MDC infected cell after treatment with pronase for 30 min then digested with RNase as in Fig. 24. No detectable changes can be observed in the appearance of the proteolytic enzyme digested inclusions (compare with Fig. 16). X 6,500

Electron Microscope Autoradiography of thin sections

1. Location of tritiated thymidine

Monolayers of MDC cells were prepared and infected with ICL virus. At intervals of 11, 15, and 18 hr post infection, the cultures were labelled with tritiated thymidine for 1 hr. The cells were washed, fixed and embedded in GMA. Thin sections were cut and prepared for autoradiography (see Materials and Methods). The specimens were exposed in a light proof box at 4°C for 2 weeks. After developing and staining, the sections were examined in the electron microscope.

At all times, more than 50 cells showing distinct nuclei were examined. Uninfected cells showed the random distribution of the silver grains all over the nucleoplasm (Fig. 26). Some of the Mitochondria exhibited the location of radioactive thymidine which indicated the synthesis of DNA in these organelles. No silver grains were observed over the other parts of the cytoplasm. Not all the nuclei of uninfected cells were labelled, since a large portion of cells would not be in the S phase and would not therefore be synthesizing DNA. In contrast, all the 12 hr infected cells showed the grains located over the early inclusions (Fig. 27). Occasionally, a few grains could be observed over the chromatin clumps marginated along the nuclear membrane.

At the time of the appearance of ring form inclusions, normally at 16 hr after infection, the majority of the grains were observed over the dark staining outer parts of these inclusions with a few grains over the light staining central areas (Fig. 28). These observations indicated that the early inclusions and the ring form structures contained newly synthesized DNA, which in the following experiments was shown to be viral DNA.

At 19 hr after infection, no silver grains were seen over the dark and the light staining inclusions, indicating the absence of radioactive DNA in their composition (Fig. 29). A few virus particles were observed to have labelled DNA, but the majority of radioactivity was located over the material originating from the ring form inclusions.

When the sections of labelled cells were treated with proteolytic enzymes, then used for autoradiography, the inclusions were partially digested and the virus coat proteins were removed but the labelled material in the nucleus was not affected. The grains could be observed over the digested inclusions and over the viral DNA core (Fig. 30). However, digestion with DNase following treatment with Proteolytic enzymes removed most of the labelled DNA and no grains could be seen over the nuclear area. (Fig. 31). In contrast, no change was observed

in stain intensity and localization of labelled DNA in the control sections incubated in distilled water (Fig. 32).

In order to determine the nature of the DNA present in early inclusions and whether this DNA participated in virus maturation, the following experiment was carried out. Eleven hr after infection, the cells were labelled with tritiated thymidine for 1 hr, then the cultures were divided into two groups. In one group, the cells were fixed immediately, and in the other group, the cells were chased in fresh medium (without radioactivity) for 7 hr. After autoradiography of the cells in thin sections, labelled DNA was found to be present in the early inclusions at 12 hr post infection (Fig. 33). When the label was chased until the time of the appearance of virus particles, the grains were preferentially located over the virus particles (Fig. 34), indicating the incorporation of the DNA present in the early inclusions, into the virus particles. Only a few grains could be observed outside the viral aggregates and there was no incorporation of label into the dark and light staining inclusions.

The role of host DNA in the formation of different inclusions, and its possible incorporation into the virus particles was determined by experiments

in which the pre-infected cells were labelled with tritiated thymidine. In order that all the cells of an unsynchronized population could be labelled, the period of labelling was extended to 24 hr. The cultures were then infected with virus and incubated in fresh medium (without radioactivity). Control cells were similarly labelled and chased without infection. At 16 and 18 hr post infection, the cells were fixed and prepared for autoradiography as described before. After 3 - 4 weeks of exposure time, the autoradiograms were developed and examined in the electron microscope. The control (uninfected) cells showed a uniform distribution of label over the nucleoplasm (Fig. 35 and 37). In infected cells, the grains were located over the condensed chromatin along the nuclear membrane (Fig. 36, 38). The inclusions and virus particles were not labelled, but a few grains could be observed over the nucleoli.

This experiment indicated that after virus infection, the host DNA marginated along the nuclear membrane and apparently did not participate in the formation of inclusions and virus factory.

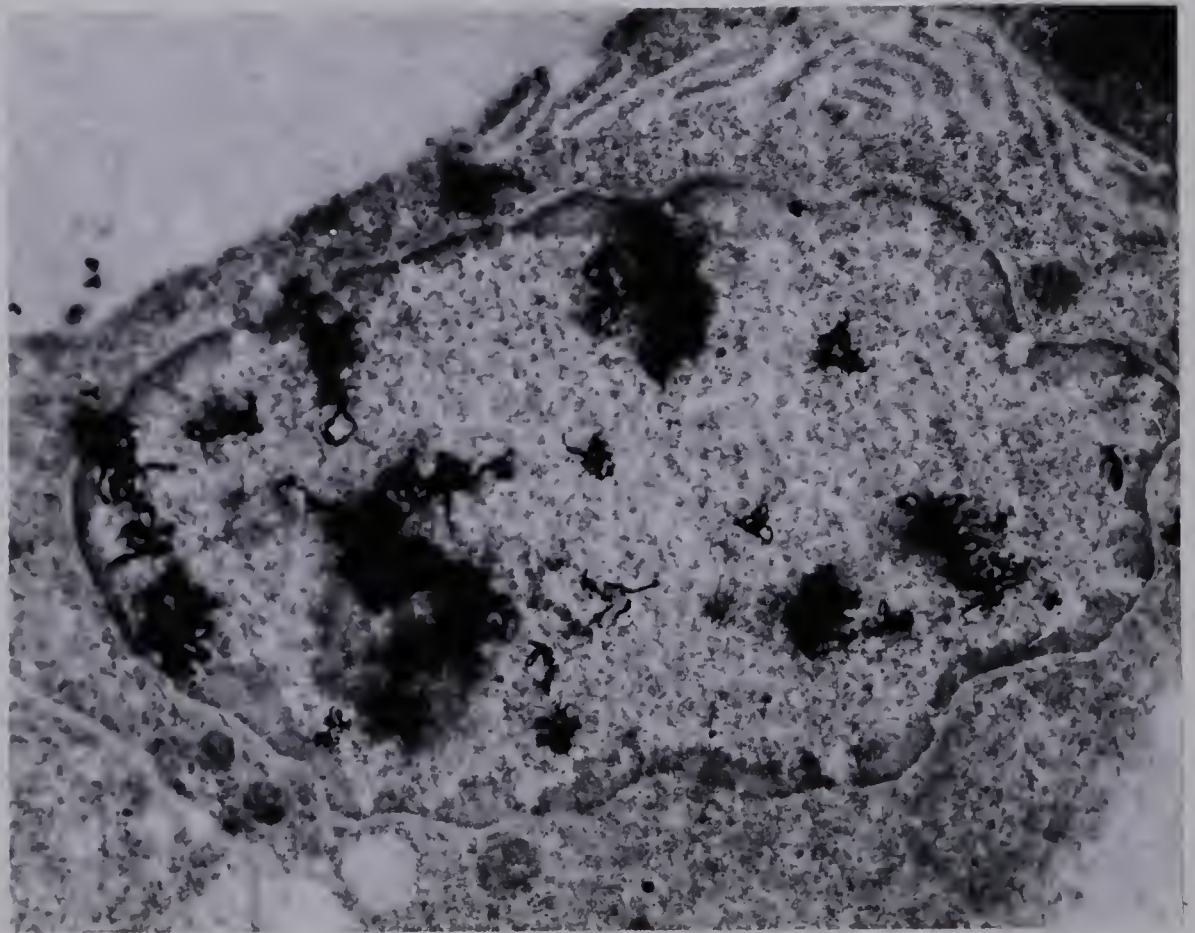
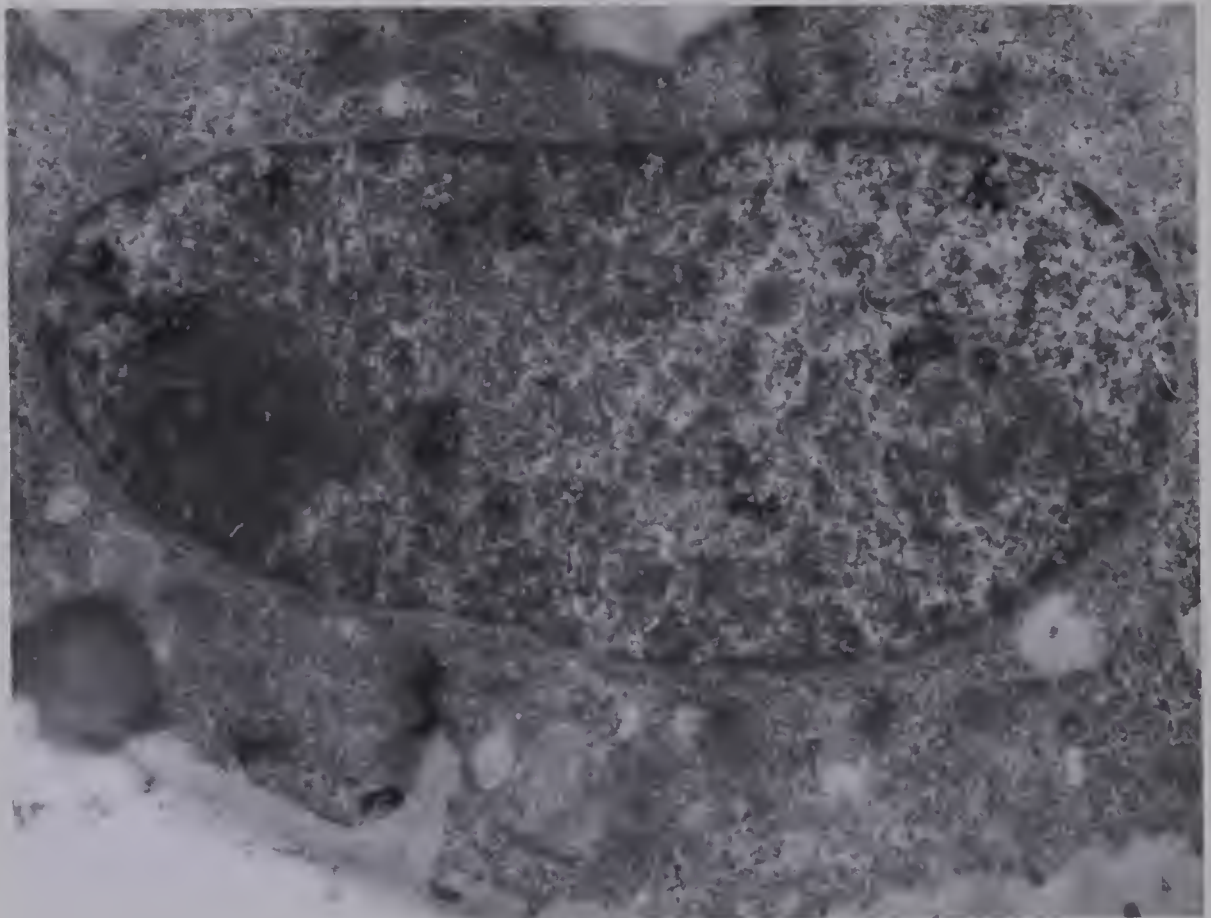


Fig. 26

Electron microscopic autoradiograph of a noninfected MDC cell labelled 1 hr with tritiated thymidine. Silver grains are located mainly over the nucleoplasm. A mitochondrion is seen to be labelled (at bottom). X 14,000

Fig. 27

Electron microscopic autoradiograph of MDC cell 12 hr after infection with ICL virus. One hr pulse with tritiated thymidine. Intense labelling of the early inclusions. Some grains are seen over the chromatin clumps marginated toward the nuclear membrane. X 16,000

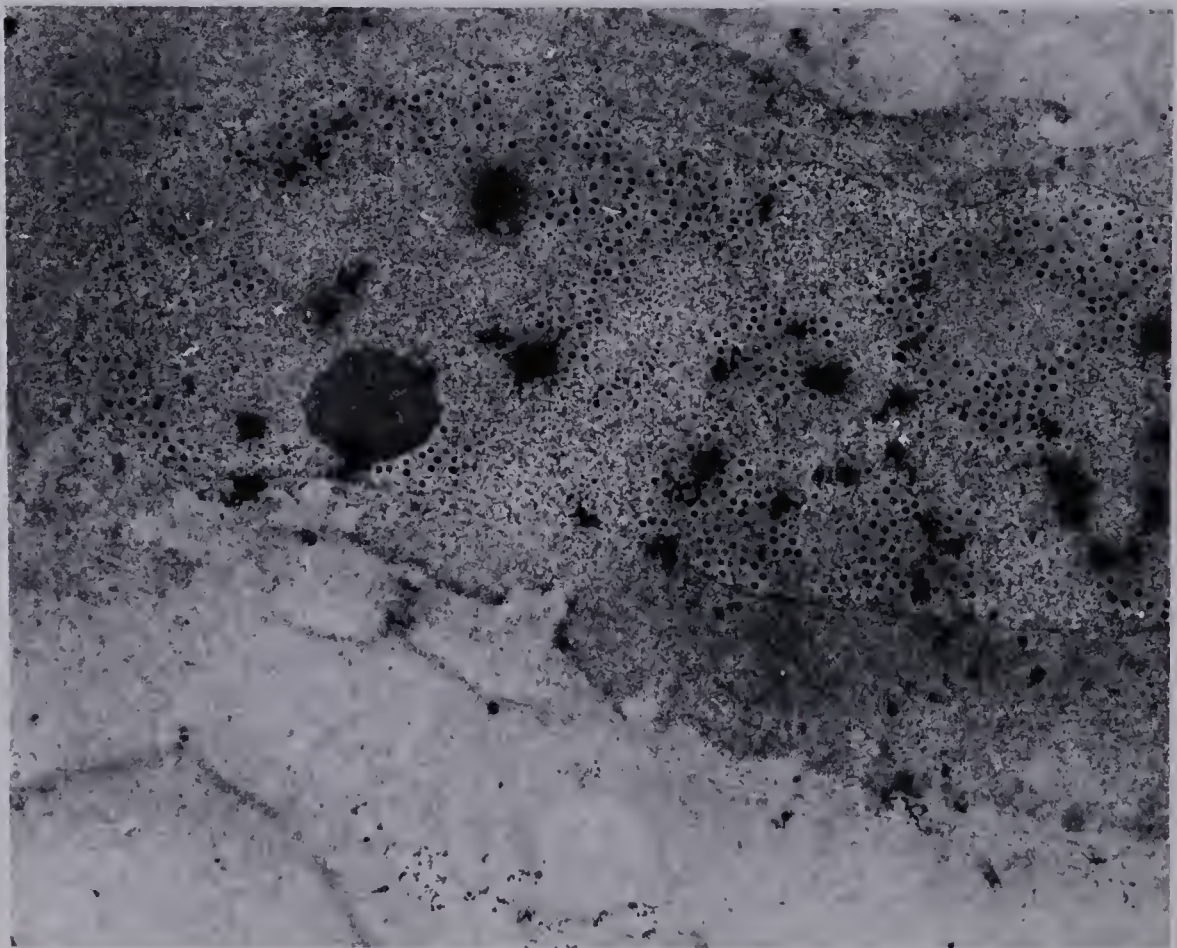
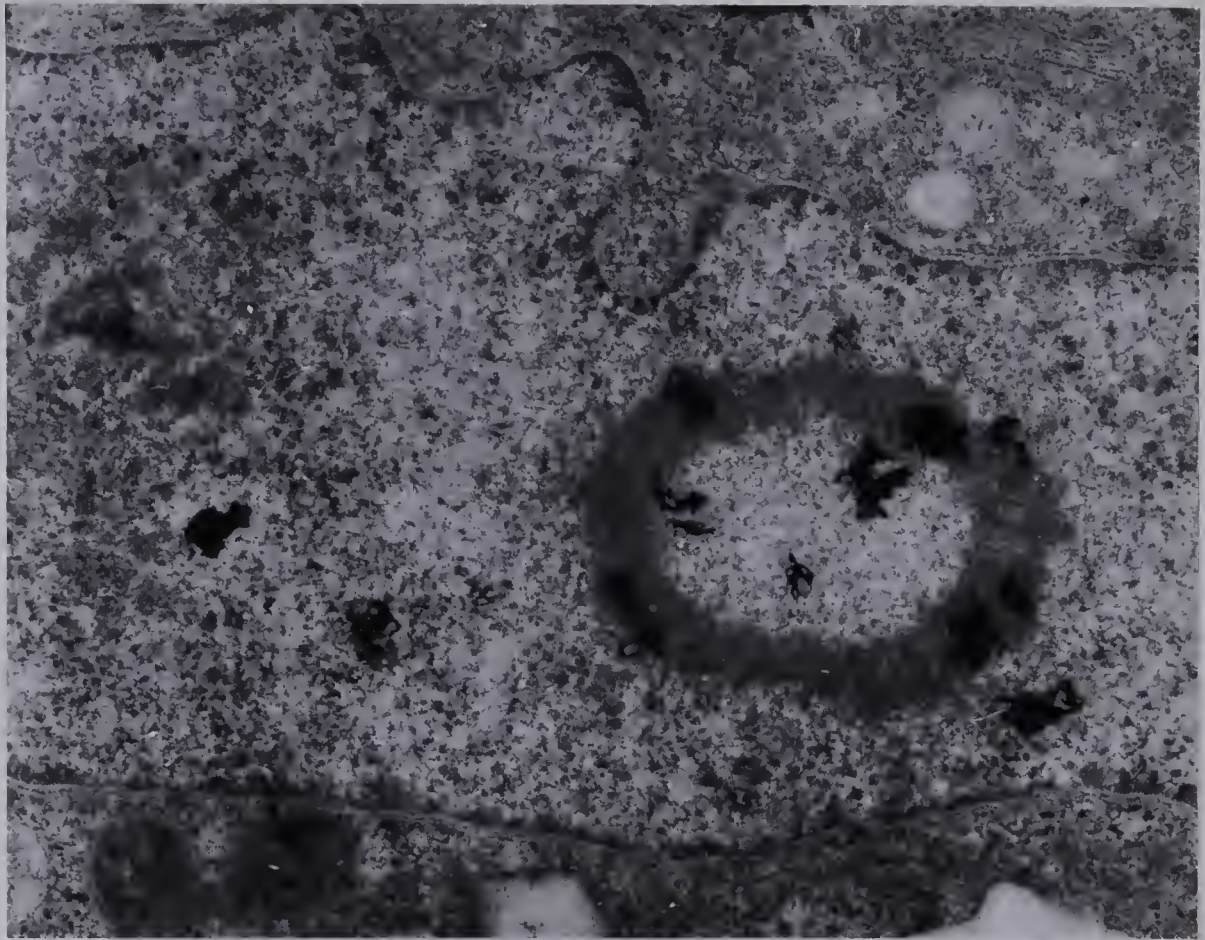


Fig. 28

Electron microscopic autoradiograph of MDC cell 16 hr after infection.

One hr pulse with tritiated thymidine. The silver grains are preferentially located over the dark staining material of ring form inclusion. X 14,000

Fig. 29

Electron microscopic autoradiograph of MDC cell 19 hr after infection and labelled with radioactive thymidine for 1 hr.

Dark and light staining inclusions are not labelled. Silver grains are located over the fibrogranular mass and over the virus particles. X 8,000

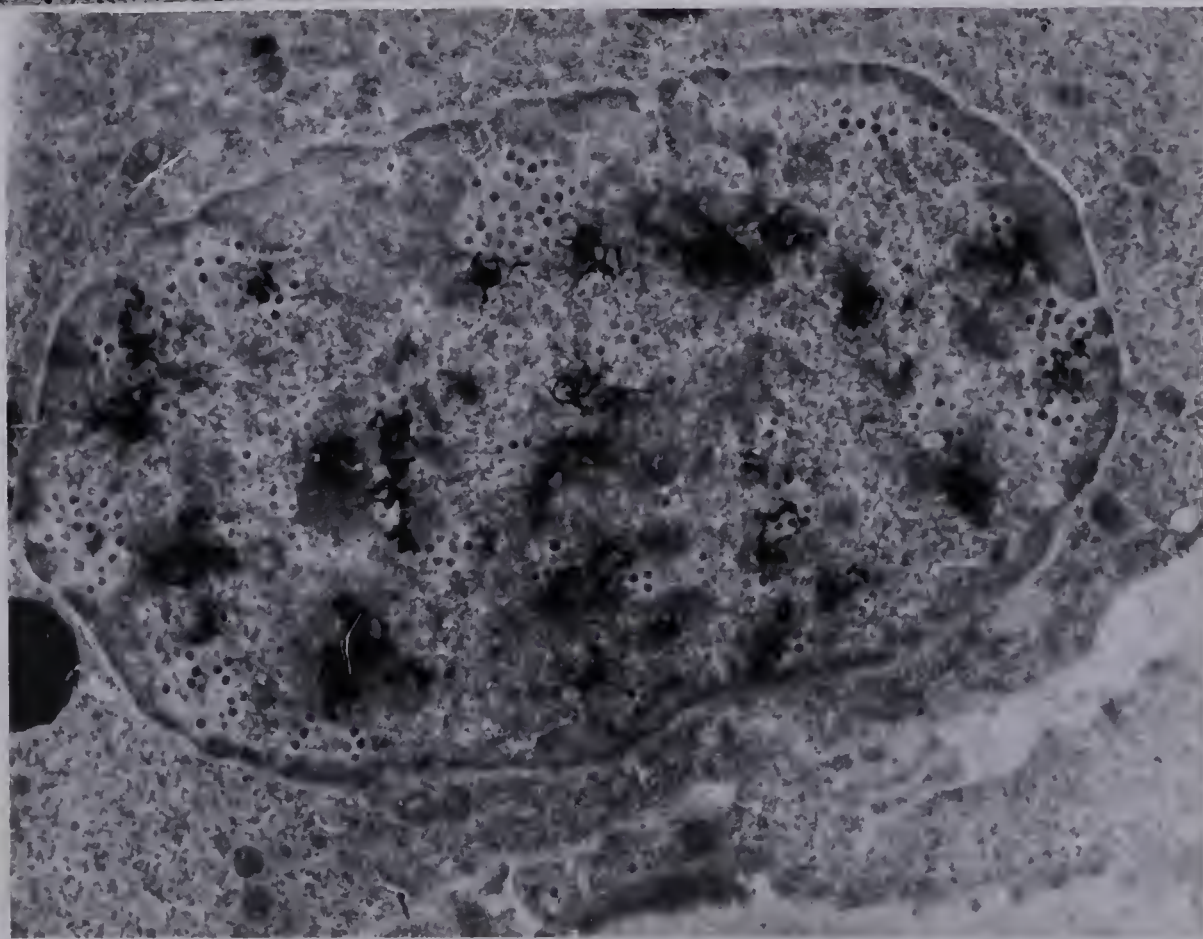
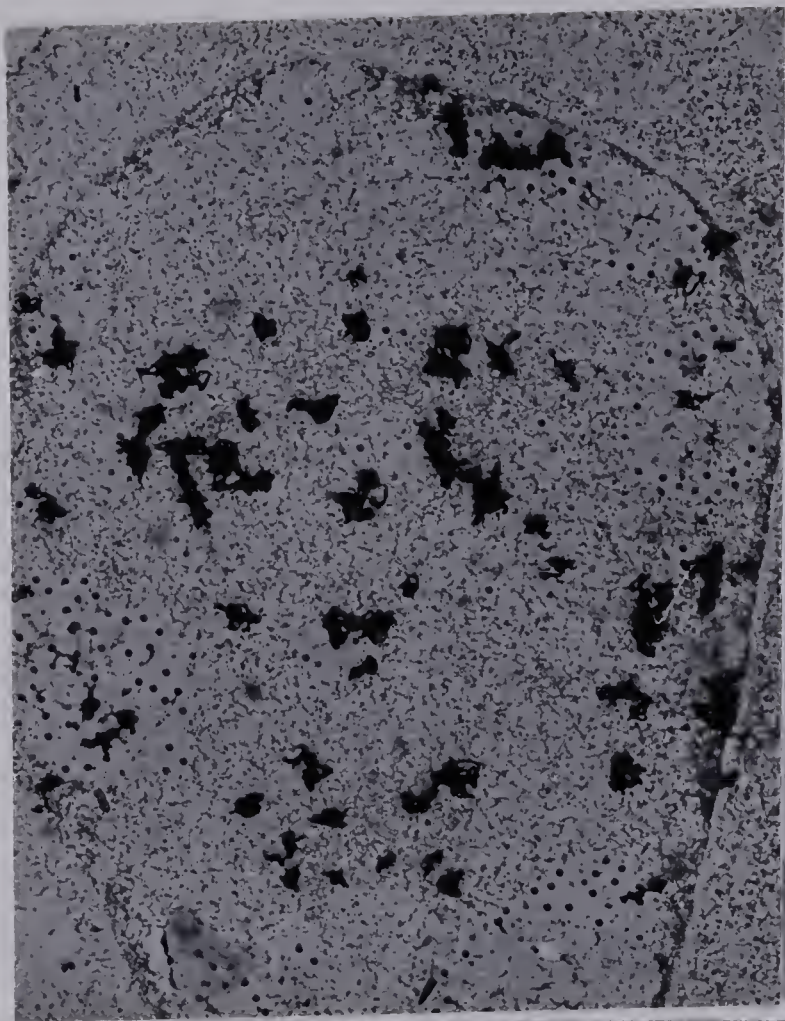


Fig. 30 (top left)

MDC cell 19 hr after infection and labelled as in Fig. 29. The section was digested with pronase for 30 min and then used for autoradiography. The inclusion (fibrogranular central mass) is partially digested. The viral coat proteins are removed. The labelled material is not removed and the grains are seen over the digested inclusions and over the virus particles. X 11,000

Fig. 31 (top right)

Electron microscopic autoradiograph of 19 hr infected MDC cell labelled with tritiated thymidine. The sections were treated with pronase then digested with DNase. The labelled material is completely removed. X 13,000

Fig. 32 (bottom)

Electron microscopic autoradiograph of infected MDC cell labelled with tritiated thymidine. The section was floated on distilled water for 2 hr. No change in the location of the labelled material can be observed. X 11,000

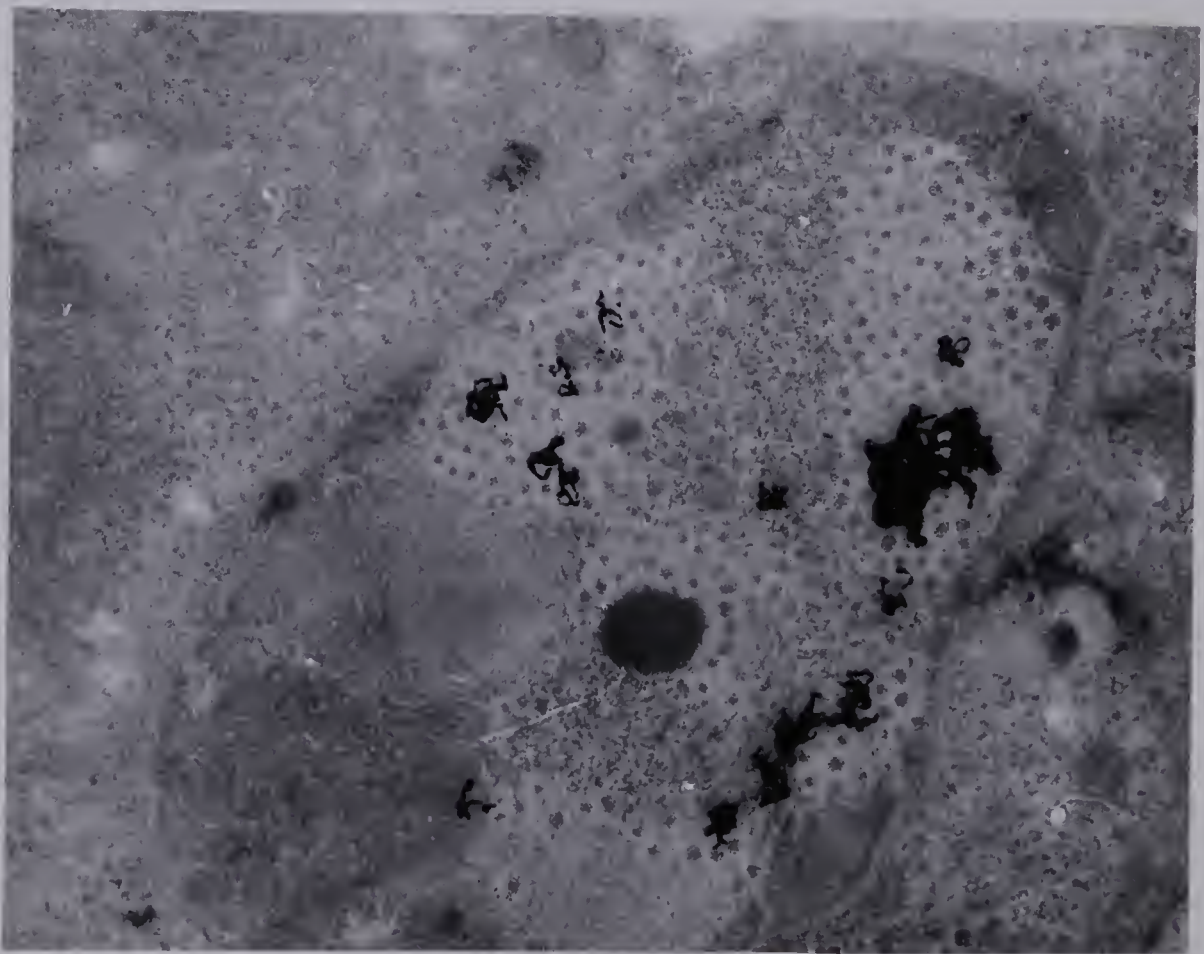
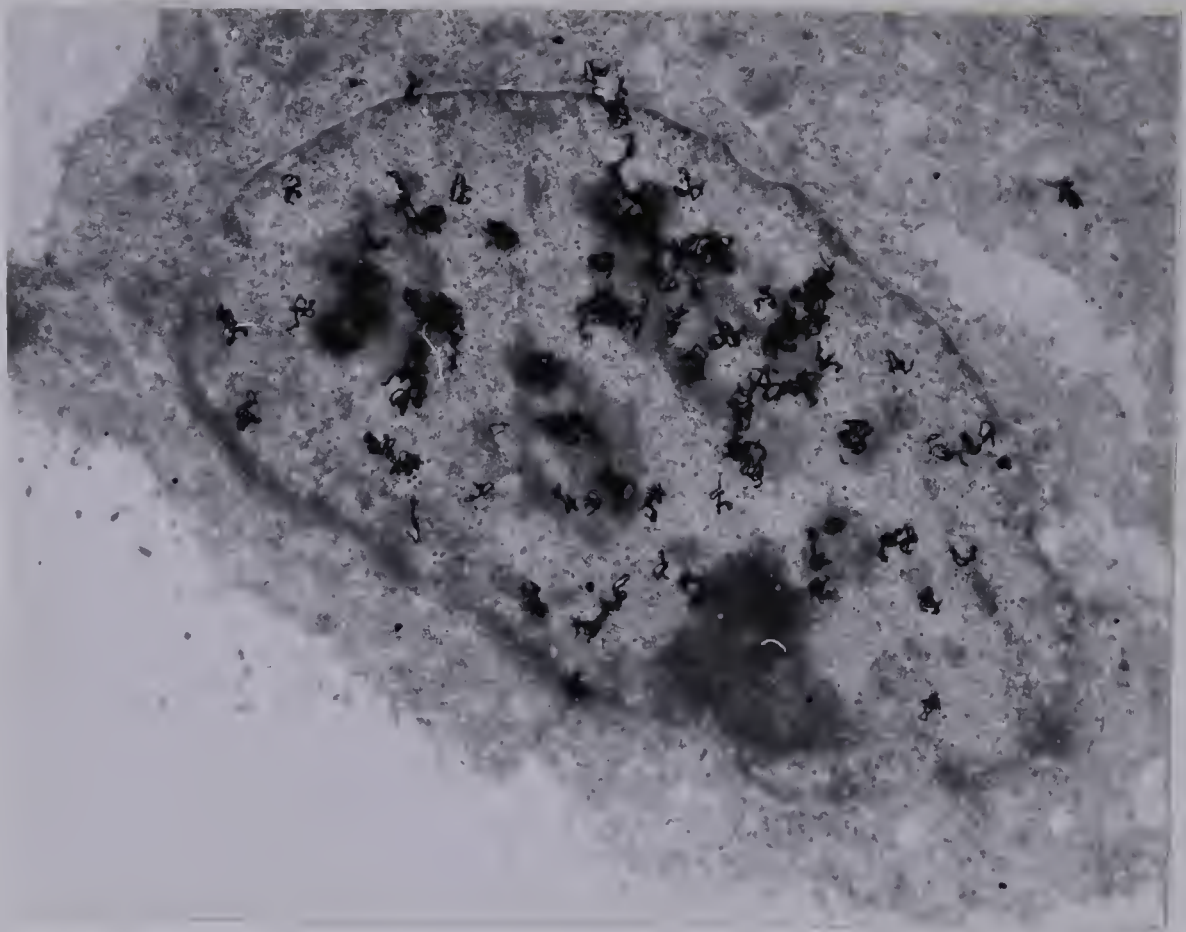


Fig. 33

Electron microscopic autoradiograph of MDC cell 12 hr after infection.

One hr pulse with tritiated thymidine. The label is mainly associated with early inclusions. A few grains are seen over the nuclear membrane. X 8,500

Fig. 34

Electron microscopic autoradiograph of infected MDC cell. The cell was labelled with tritiated thymidine as in Fig. 33, then chased in fresh medium for 7 hr. The silver grains are mainly located over the virus particles. X 11,500

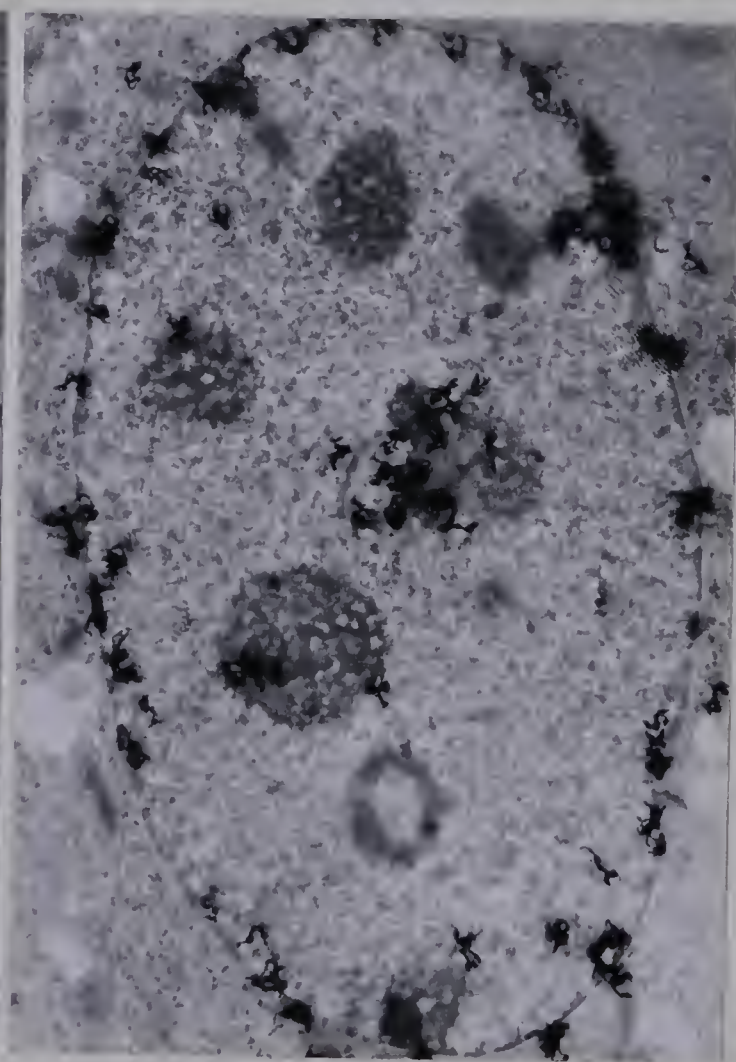
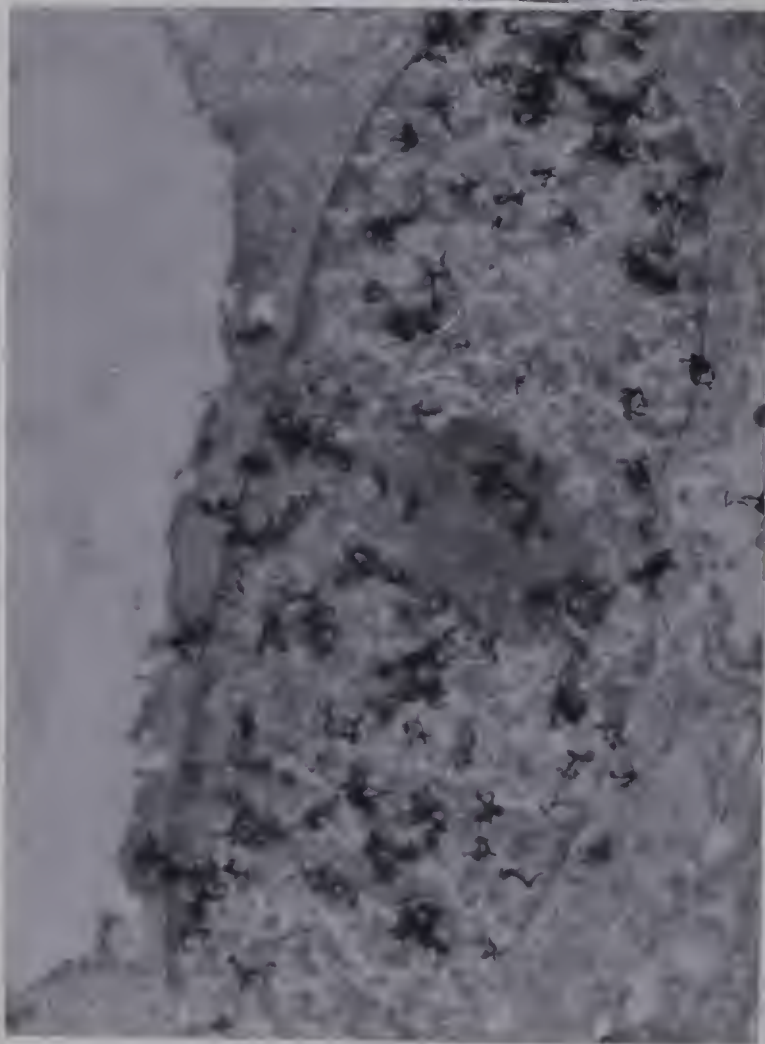
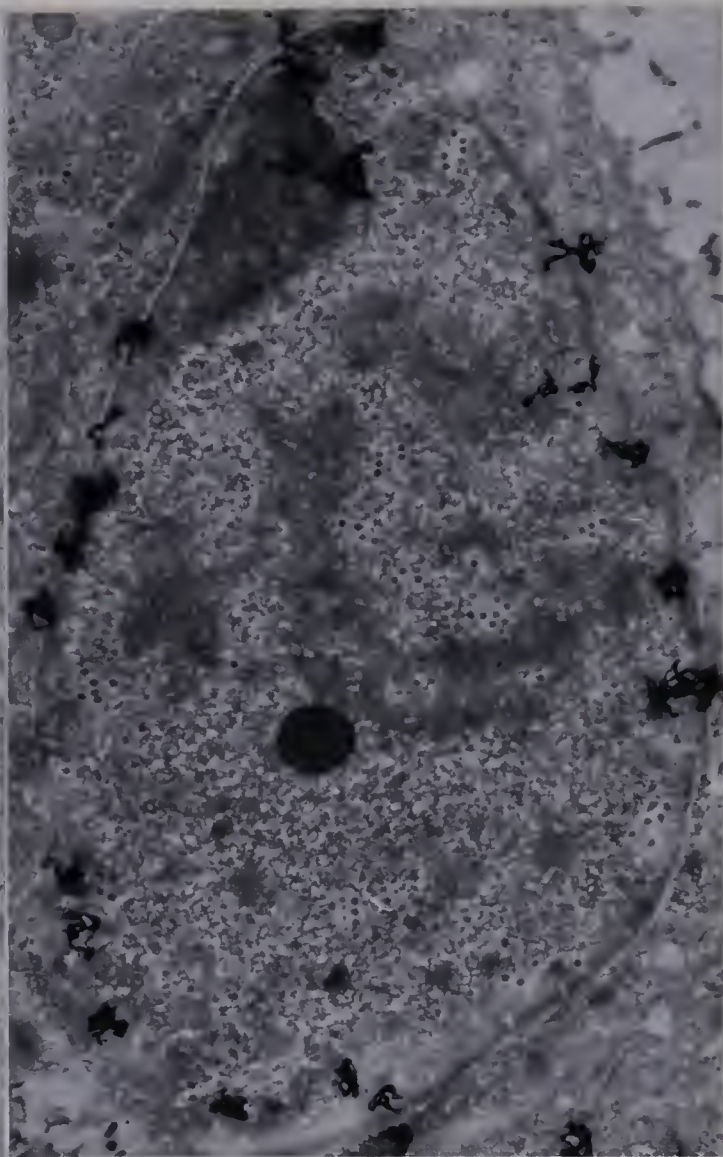
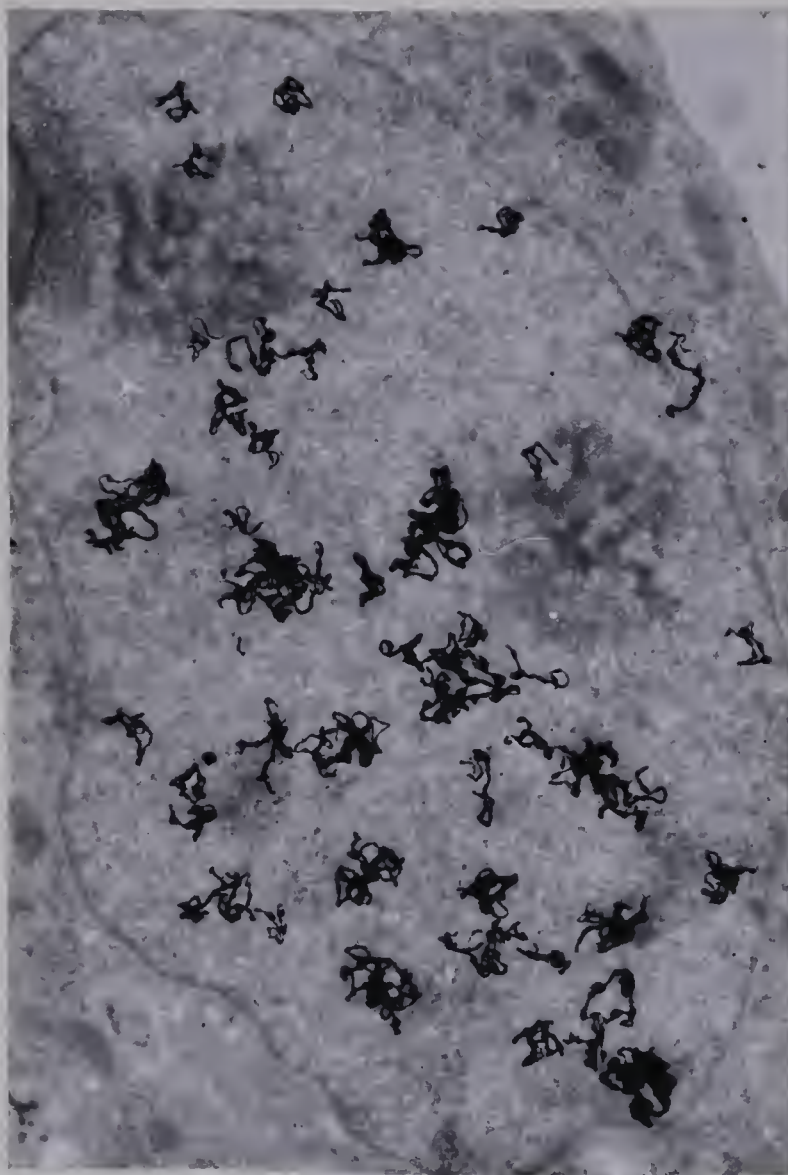


Fig. 35 (top left)

Electron microscopic autoradiograph of uninfected MDC cell labelled with tritiated thymidine for 24 hr. The silver grains are evenly distributed over the nucleoplasm. A few grains are located over the nuclear membrane. X 15,000

Fig. 36 (top right)

Electron microscopic autoradiograph of MDC cell labelled with tritiated thymidine as in Fig. 33. The cell was infected with ICL virus and incubated in fresh medium for 18 hr. The prelabelled host DNA is margined toward the nuclear membrane. The inclusions and virus particles are unlabelled. Exposure time 3 weeks. X 11,500

Fig. 37 (bottom left)

Uninfected MDC cell treated similarly to that in Fig. 35. Exposure time 1 month. X 10,000

Fig. 38 (bottom right)

16 hr after infection of prelabelled MDC cell. The prelabelled host DNA is margined toward the nuclear membrane. Nucleoli are also labelled. The ring form inclusion is unlabelled. Exposure time 1 month. X 8,500

2. Location of tritiated uridine

Control (uninfected) and infected cells were labelled with tritiated uridine in the presence of 20 times excess of cold thymidine at the intervals of 11, 15, and 18 hr after virus infection. The time of labelling was either 10 or 30 min at 37°C. The autoradiography procedure was performed as described before (see Materials and Methods) with the exception of exposure time which was 3 weeks for the 30 min labelled cells, and 1 month for the 10 min labelled cells. More than 50 cells in which the inclusions could be observed were examined. The uninfected cells labelled for 30 min showed heavily labelled nucleoli (Fig. 39). A few grains could be observed over the nucleoplasm and cytoplasm. The control cells labelled for 10 min showed the same location of label but because of the short time of radioactive incorporation, only a small number of reduced silver grains could be visualized (Fig. 40).

At 12 and 16 hr after infection, the cells labelled for 30 min showed the grains located mainly over the nucleoli and the inclusions (Fig. 41). The same pattern of distribution of the silver grains was observed over the cells labelled for a short time (10 min), but in general, only a few grains could be seen over the inclusions and the

nucleoli (Fig. 42 and 43).

Twenty hr after infection, the time when the late inclusions (dark and light staining inclusions) and a large number of virus particles are formed, the nucleoli or infected cells exposed to tritiated uridine for 30 min were labelled indicating the continuous RNA synthesis in these organelles at this stage of infection (Fig. 44). However, the dark and the light staining inclusions were not labelled (Fig. 45).

When the sections of control and infected cells, labelled with radioactive uridine were digested with 0.1% RNase, then used for autoradiography, it was found that the enzyme did not attack the radioactive material present in the nuclear components, and the grains could be seen over the nucleoli and inclusions (Fig. 46). Proteolytic enzyme digestion of thin sections resulted in the partial digestion of inclusions but the labelled material was unaffected.

Digestion of the thin sections with RNase following treatment with proteolytic enzymes resulted in the removal of most of the radioactive material from the labelled cells and almost no silver grains were observed over the digested sections (Fig. 47 and 48). This experiment indicated that the reduced silver grains observed over the tritiated uridine labelled cells was due to the presence of radioactive

RNA in the labelled organelles.

In order to determine whether the tritiated uridine taken up by the cells was incorporated into RNA, and to estimate the amount of radioactivity incorporated into DNA, the control and infected cells were exposed to medium containing tritiated uridine in the presence of a 20-fold excess of cold thymidine for 10 and 30 min. Then the cells were washed in ice cold medium and disrupted by freezing and thawing. The total counts incorporated into the cells and into the acid soluble material was determined as described in Materials and Methods. RNA and DNA were extracted according to the method of Schmidt and Thannhauser (see Materials and Methods), and samples were counted in a liquid scintillation counter.

Table II indicates that in infected cells labelled for 10 min, about 7.6% of the counts were found to be incorporated into the DNA. When the time of labelling extended to 30 min, the incorporation was only slightly increased (10.2%). Almost the same amount of radioactivity was found in the DNA of uninfected cells, but the total counts incorporated into the infected cells were higher than those found in uninfected cells (Table II).

TABLE II
INCORPORATION OF ³H-URIDINE INTO DNA AND RNA

Time of Incorporation	C.P.M. Incorporated				% Incorporation		
	Total	Acid-sol.	RNA	DNA	Acid-Sol.	RNA	DNA
Infected:							
10 min.	905	140	711	70	15	77.8	7.6
30 min.	2,410	240	1,991	254	9.6	80.2	10.2
Uninfected:							
30 min.	1,920	275	1,420	250	19.2	68	12.8

Twelve hr after infection, the cells were labelled with ³H-uridine for 10 and 30 min. Uninfected cells were labelled with the same precursor for 30 min. RNA and DNA extraction was described in Materials and Methods. The percent incorporation was calculated from the total counts of the fractions.

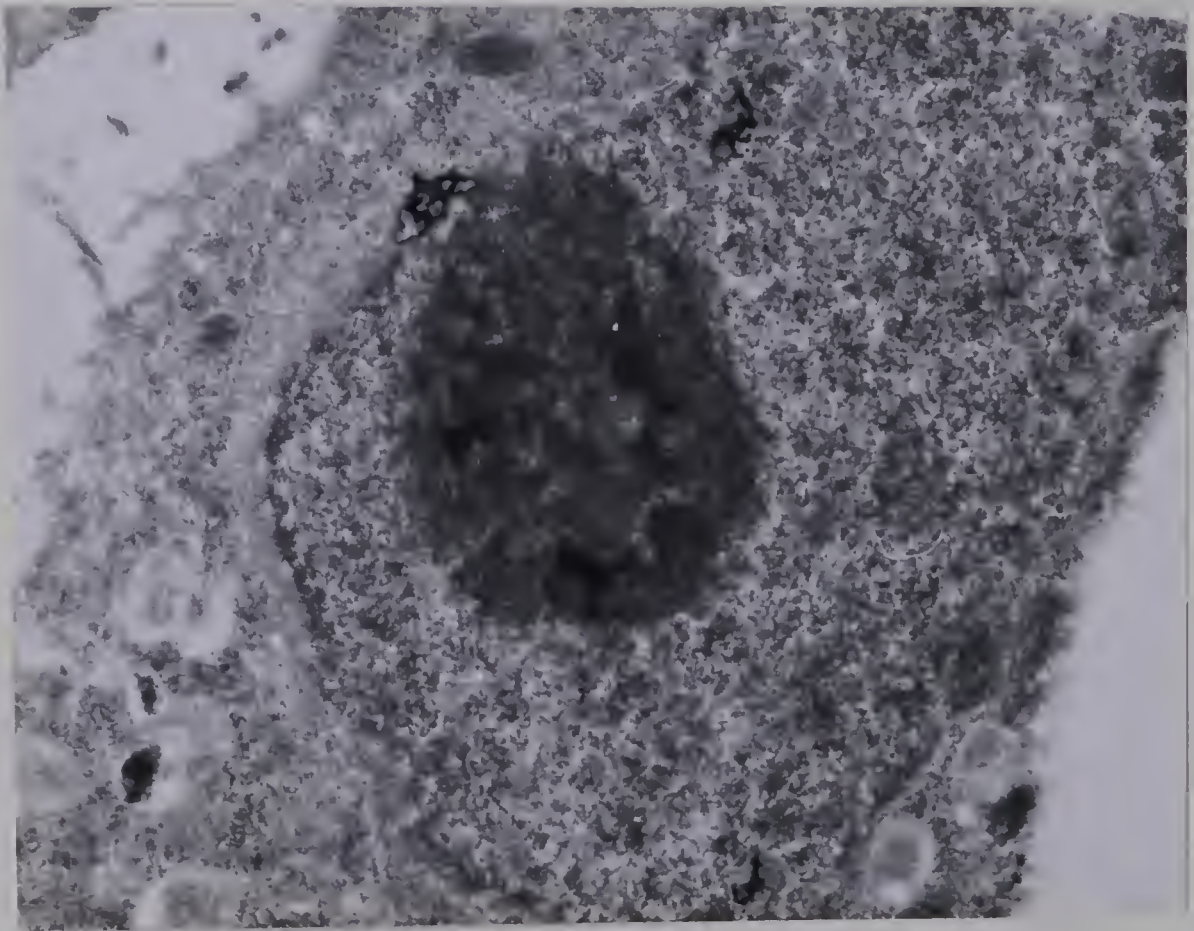
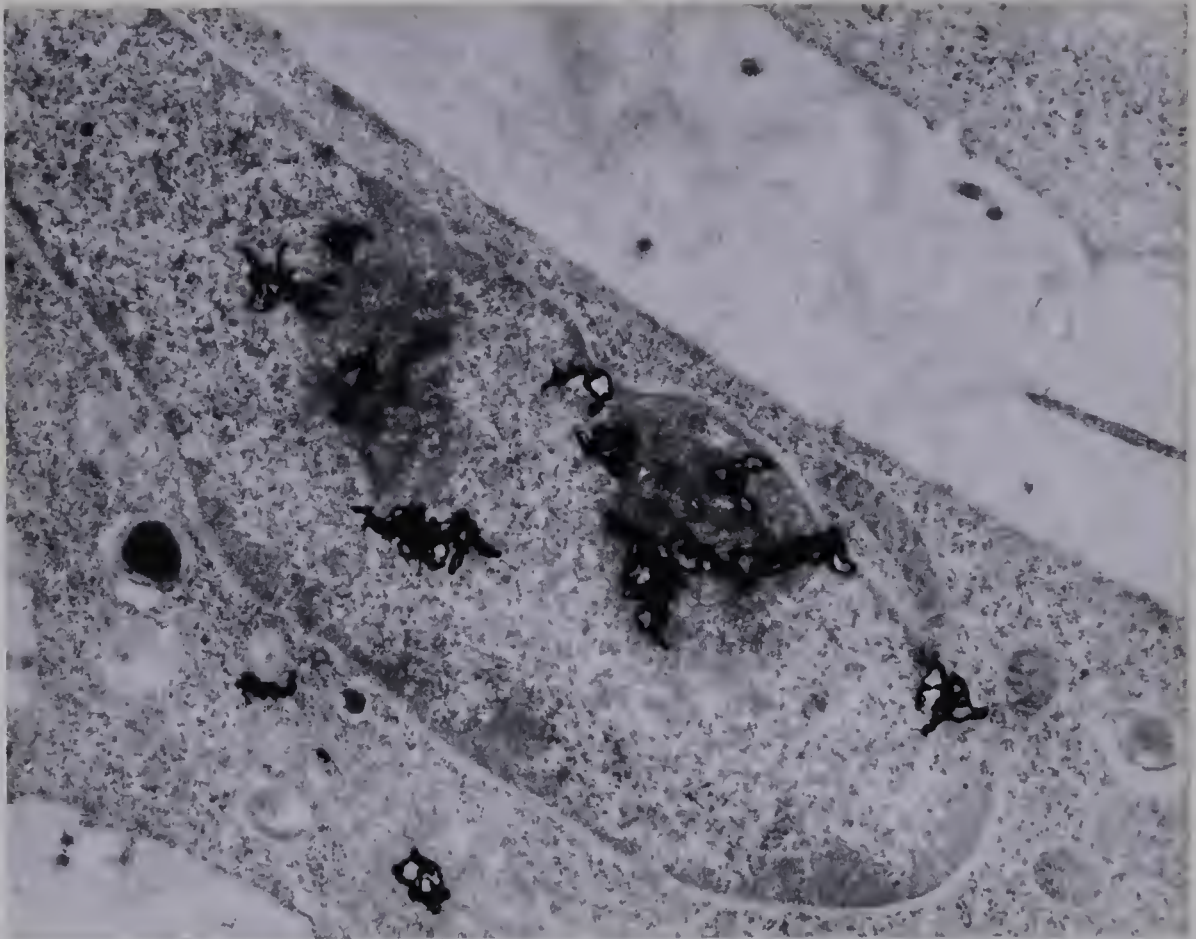


Fig. 39

Electron microscopic autoradiograph of uninfected MDC cell labelled with tritiated uridine for 30 min. The nucleoli are heavily labelled. A few grains are seen over the cytoplasm. X 12,500

Fig. 40

Similar autoradiograph to that in Fig. 39. The cell was labelled with tritiated uridine for 10 min. The silver grains are mainly located over the nucleolus. X 16,000

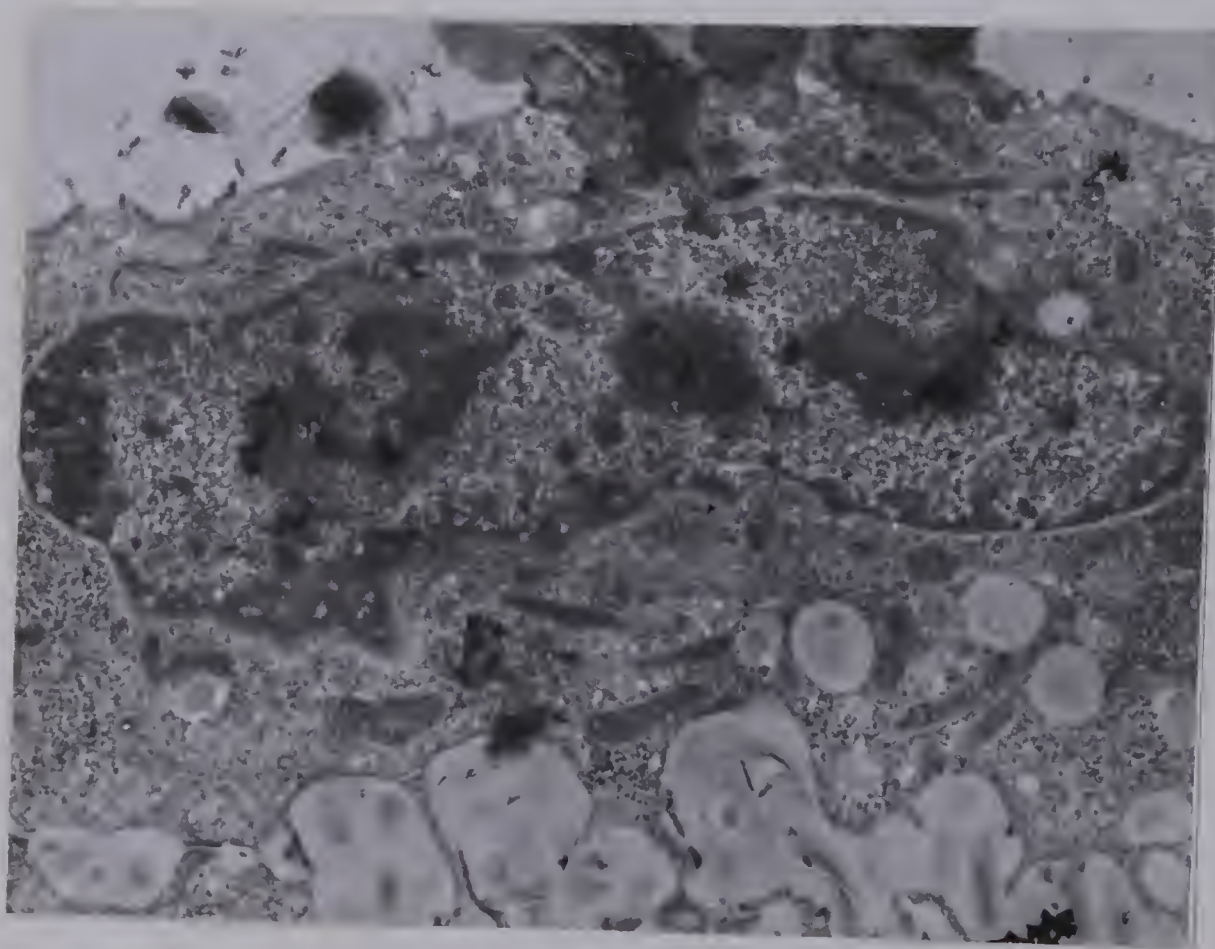
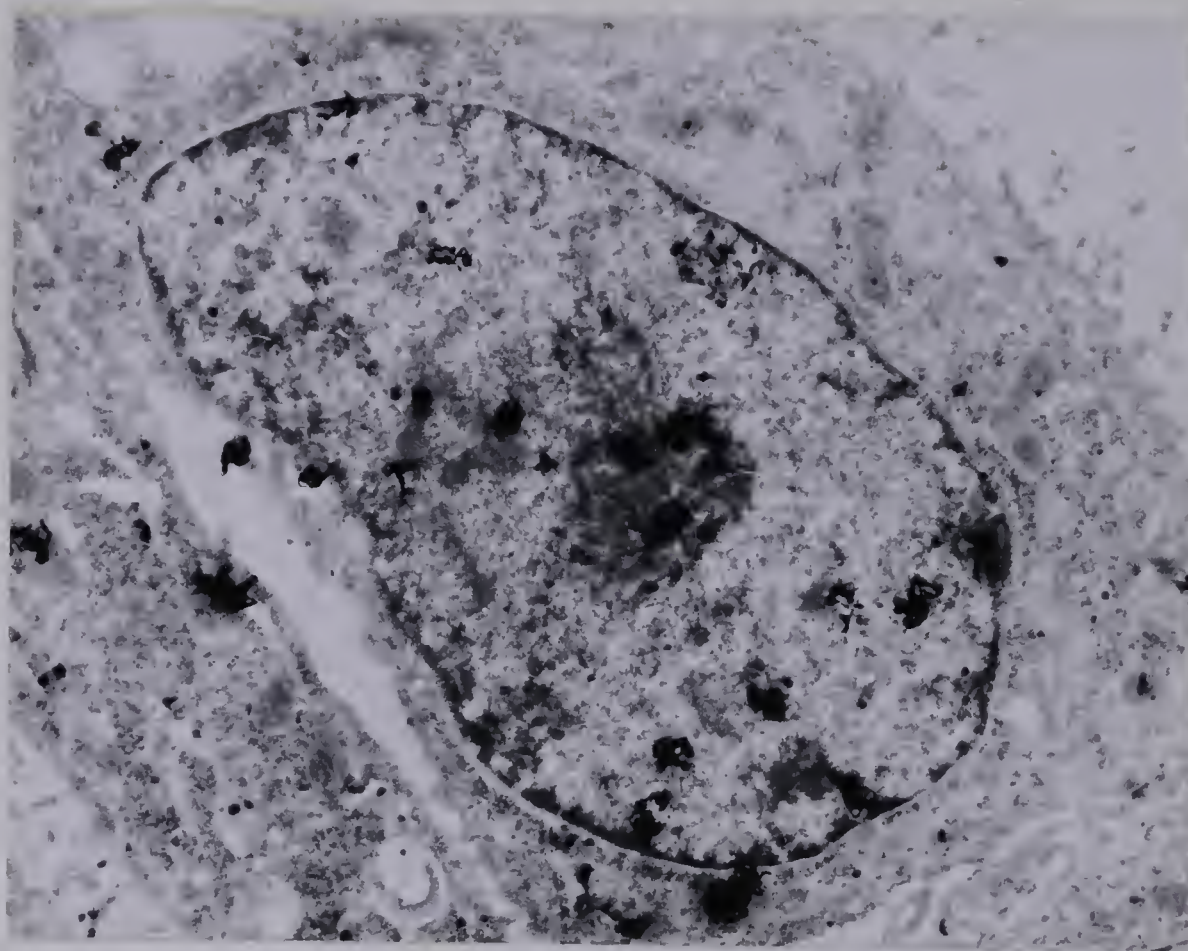


Fig. 41

Electron microscopic autoradiograph of MDC cell 12 hr after infection. 30 min pulse with tritiated uridine. Nucleolus and early inclusions are labelled. A few silver grains are seen over the nucleoplasm and cytoplasm. Exposure time 3 weeks. X 10,500

Fig. 42

The same autoradiograph as in Fig. 41. Pulsing time was 10 min. Exposure time 1 month. The silver grains are mainly located over the early inclusions and over the nucleolus. X 7,000

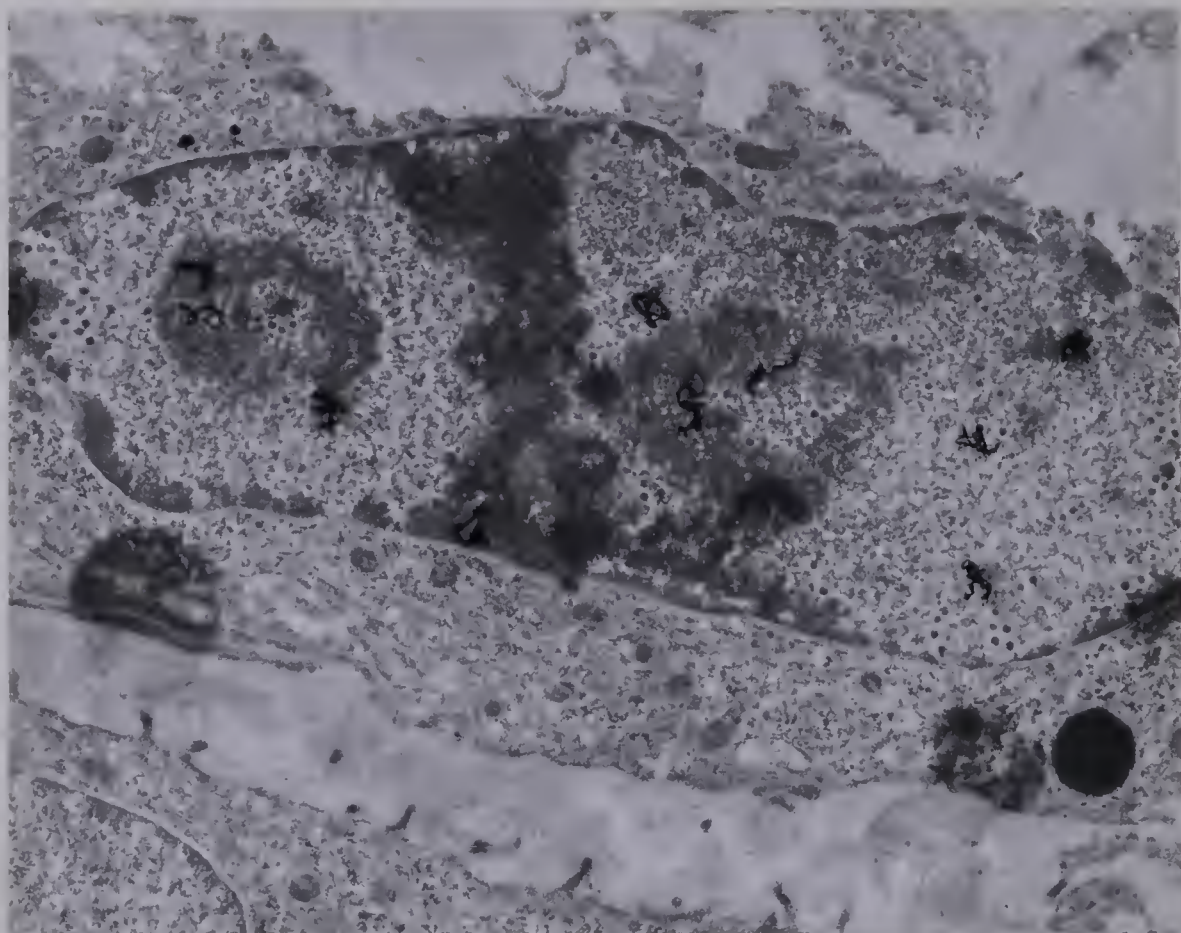


Fig. 43

Electron microscopic autoradiograph of MDC cell 16 hr after infection.

10 min pulse with tritiated uridine. The silver grains are preferentially located over the ring form inclusions. The nucleolus is seen at the middle. Exposure time 1 month. X 11,000

Fig. 44

Electron microscopic autoradiograph of MDC cell 18 hr after infection.

30 min pulse with tritiated uridine. Nucleolus is heavily labelled. No silver grains can be seen over the light staining inclusions and virus particles. Exposure time 3 weeks. X 32,000

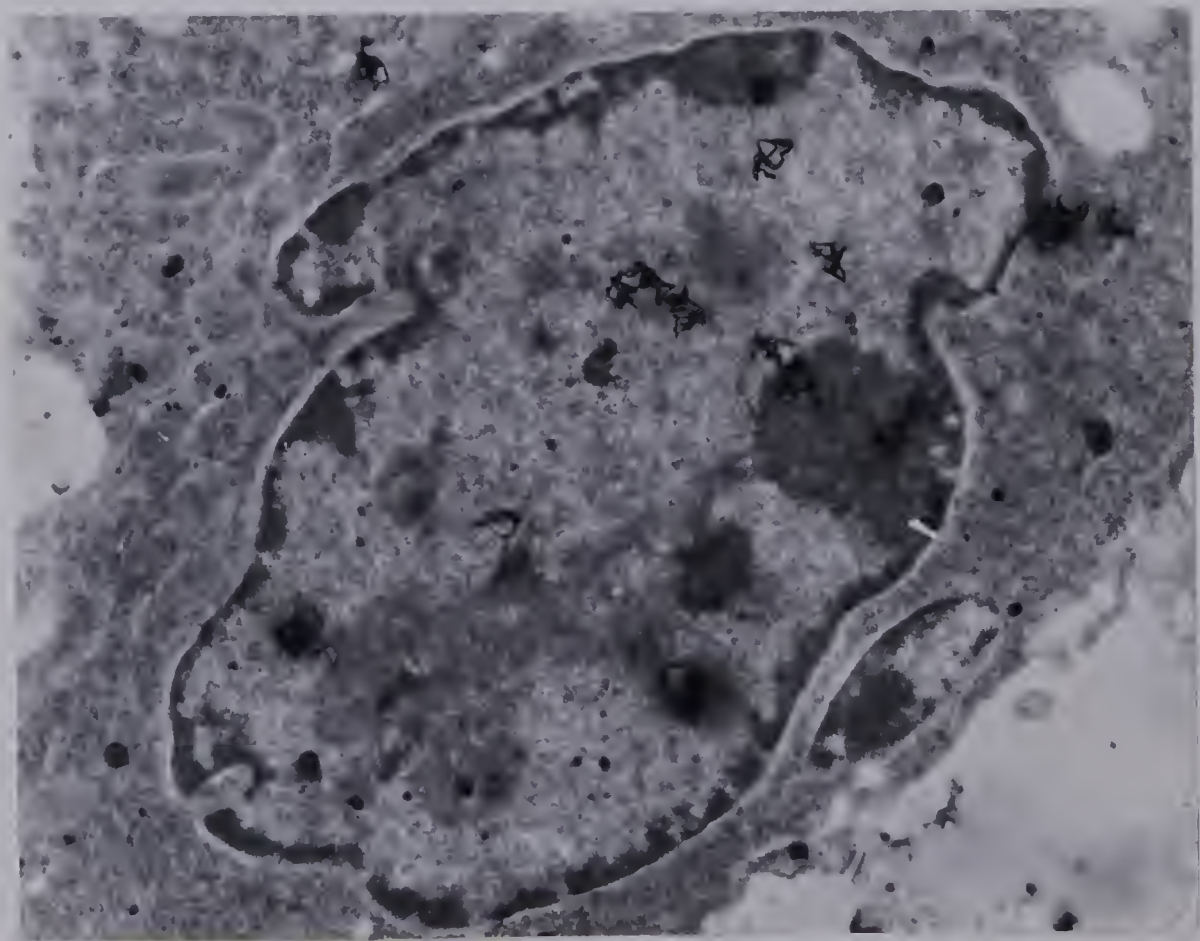
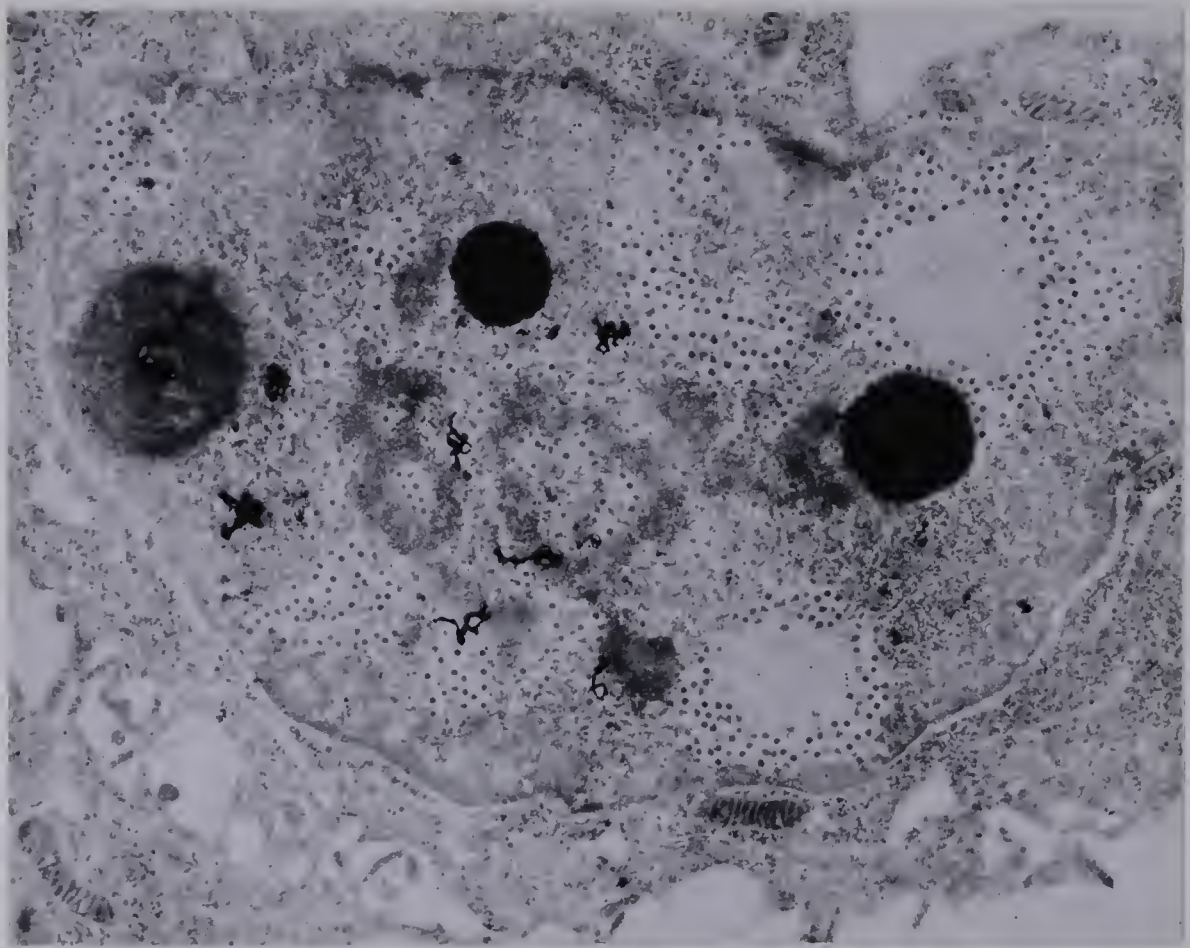


Fig. 45

The same autoradiograph as in Fig. 44.
None of the dark and light staining inclusions are
labelled with tritiated uridine. X 14,000

Fig. 46

Electron microscopic autoradiograph of infected MDC cell
labelled with tritiated uridine for 30 min. The section
was floated on 0.1% RNase solution for 2 hr, then used
for autoradiography. The enzyme did not attack the
labelled material. The grains are seen over the nucleolus
and over the inclusions. X 11,000

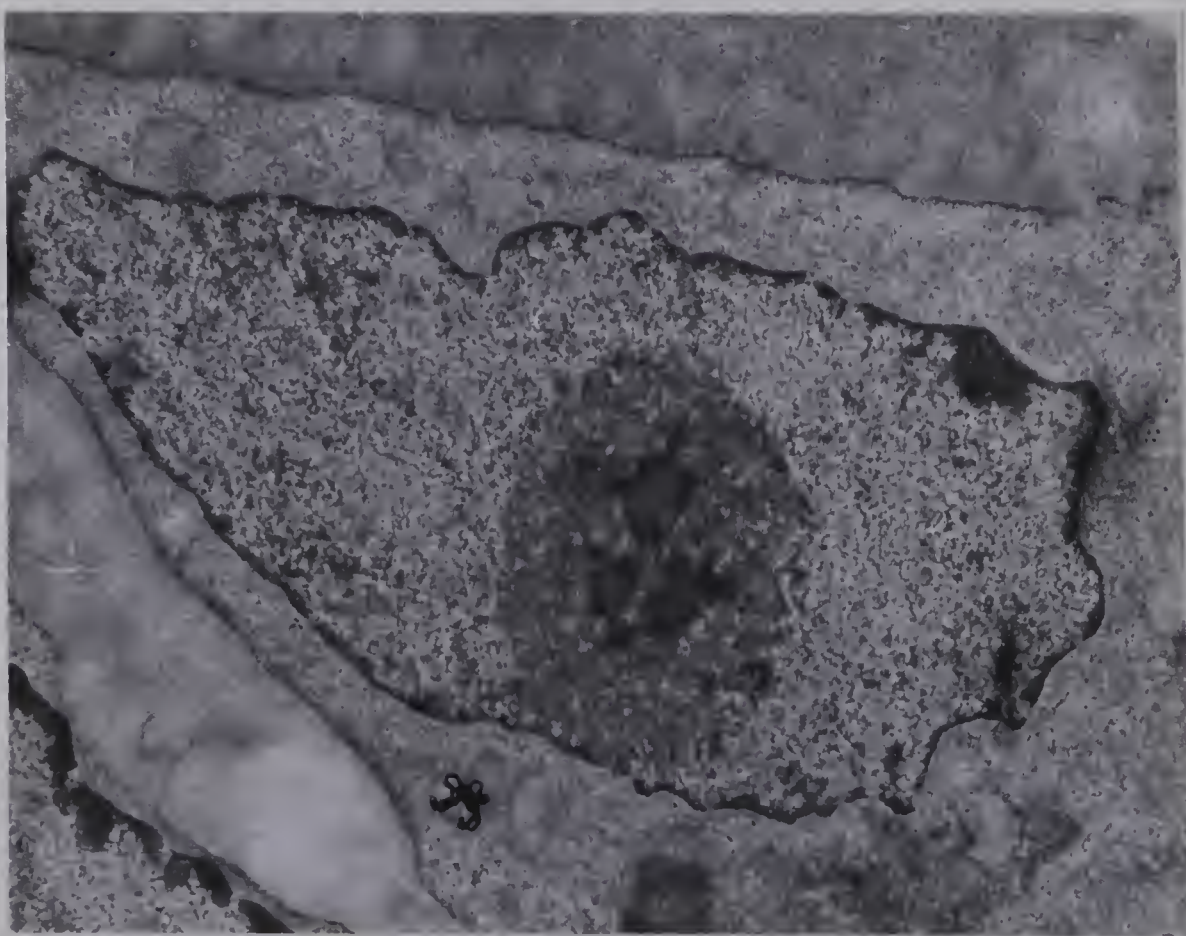
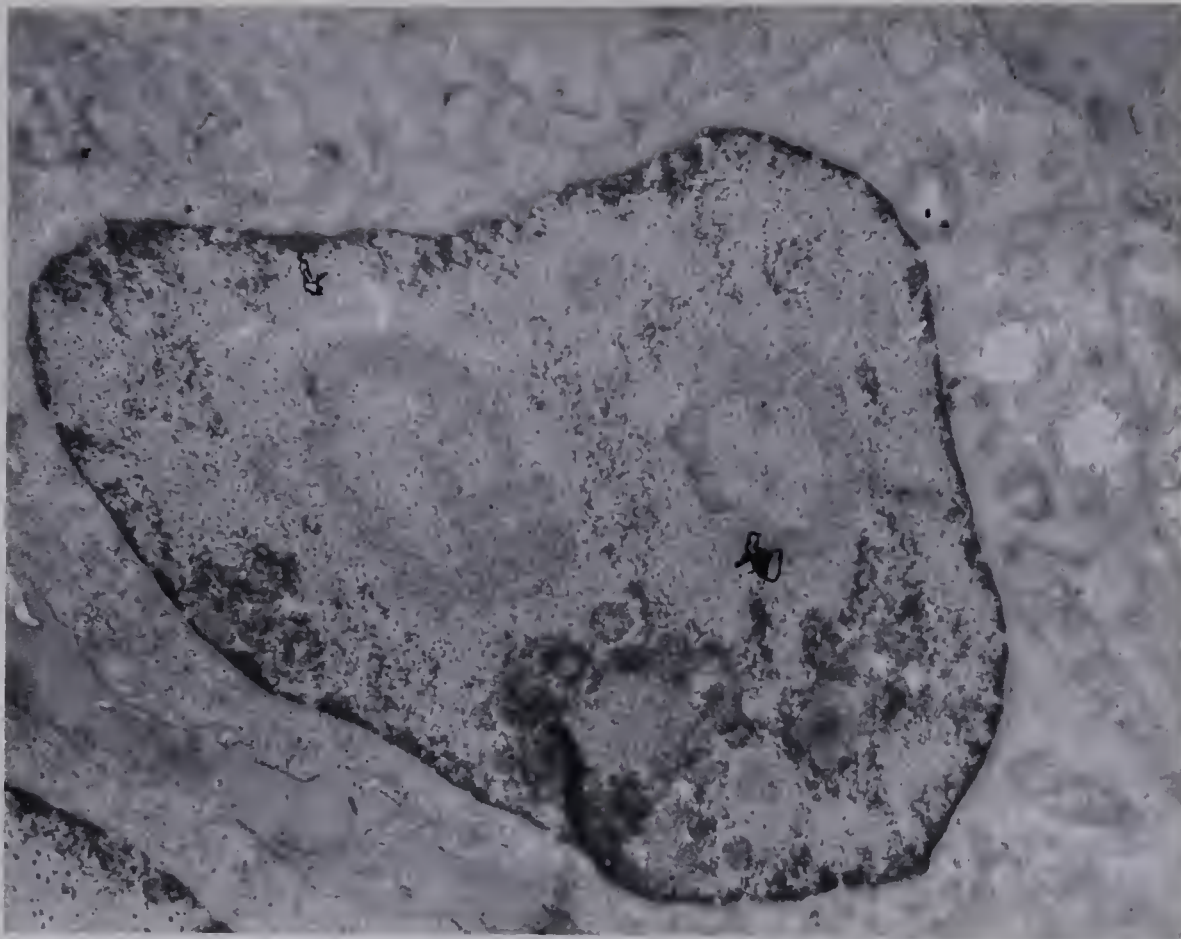


Fig. 47

Electron microscopic autoradiograph of infected MDC cell labelled with tritiated uridine for 30 min. The section was treated with pronase for 30 min then digested with 0.1% RNase for 1 hr. The radioactive material is removed from the ring form inclusions and nucleoli. A few silver grains which can be seen are probably due to the insufficient washing of the digested sections. X 13,000

Fig. 48

Autoradiograph of uninfected MDC cell labelled with radioactive uridine for 30 min and treated with enzymes as in Fig. 47. All the radioactive material is removed from the nucleolus (compare with Fig. 39). X 14,000

3. Location of tritiated leucine

At 11, 15, and 18 hr after virus inoculation, the cells were pulse labelled with tritiated leucine for 30 min, washed in ice cold medium, fixed in glutaraldehyde, and embedded in GMA. Autoradiography of thin sections, with an exposure time of two weeks, was performed as described in Materials and Methods.

Electron microscopic examination of 50 cells in which the inclusions could be observed, revealed the location of reduced silver grains distributed over the nucleus and cytoplasm of 12 and 16 hr infected cells (Fig. 49), but preferentially, over the early and ring form inclusions.

Seventeen hr after infection, the dark staining inclusions in the nucleus and cytoplasm were heavily labelled (Fig. 50 and 51). Some grains could be observed over the nucleoli, nucleoplasm, and cytoplasm with no preferential location on a particular region. There were few if any grains located over the light staining inclusions (Fig. 52).

In the experiment in which the sections of infected cells were first digested with proteolytic enzymes and then used for autoradiography, all the labelled material was removed from the digested dark staining inclusions (Fig. 53). A few grains could be seen over some of the infected cells which

were probably due to the insufficient washing of the digested material. It could therefore be concluded that the dark staining inclusions which were heavily labelled with tritiated leucine, and susceptible to digestion with proteolytic enzymes, consisted of proteins.

The light staining inclusions which at the time of appearance were not labelled with radioactive leucine, but were digestible with proteolytic enzymes, were thought to be formed from the accumulation of presynthesized proteins. In order to determine whether these proteins were the accumulation of existing cell proteins, made before the infection, or were synthesized during the infection, the following experiments were carried out:

a) MDC cells in monolayer were exposed to a medium containing tritiated leucine at 37°C for 30 min. Then the cells were washed and immediately infected with ICL virus. The cultures were incubated in fresh medium (without radioactivity) at 37°C until the time of the appearance of the dark, and the light inclusions (18 hr). Thin sections of cells were used for autoradiography (see Materials and Methods) with an exposure time of 2 - 3 weeks. In electron microscopic examination of the thin sections, it was found that the radioactive proteins, synthesized before the infection, did not incorporate into the

dark and the light staining inclusions. The reduced silver grains were randomly located over the nucleus and cytoplasm and the late inclusions (dark and light staining) appeared unlabelled (Fig. 54).

b) Twelve hr after infection, the infected cells were labelled with tritiated leucine for 30 min and then chased in fresh medium for 6 hr. After autoradiography of the thin sections, the infected cells showed the grains preferentially located over the light staining inclusions (Fig. 55) indicating that some of the protein synthesized during the 30 min at 12 hr after infection, were incorporated into the light staining inclusions. Some grains could be observed over the virus particles and over the material of the ring form inclusions. However, the dark staining inclusions either in the nucleus or in the cytoplasm were not labelled (Fig. 56 and 57), suggesting that these inclusions were not resulted from the accumulation of presynthesized proteins.

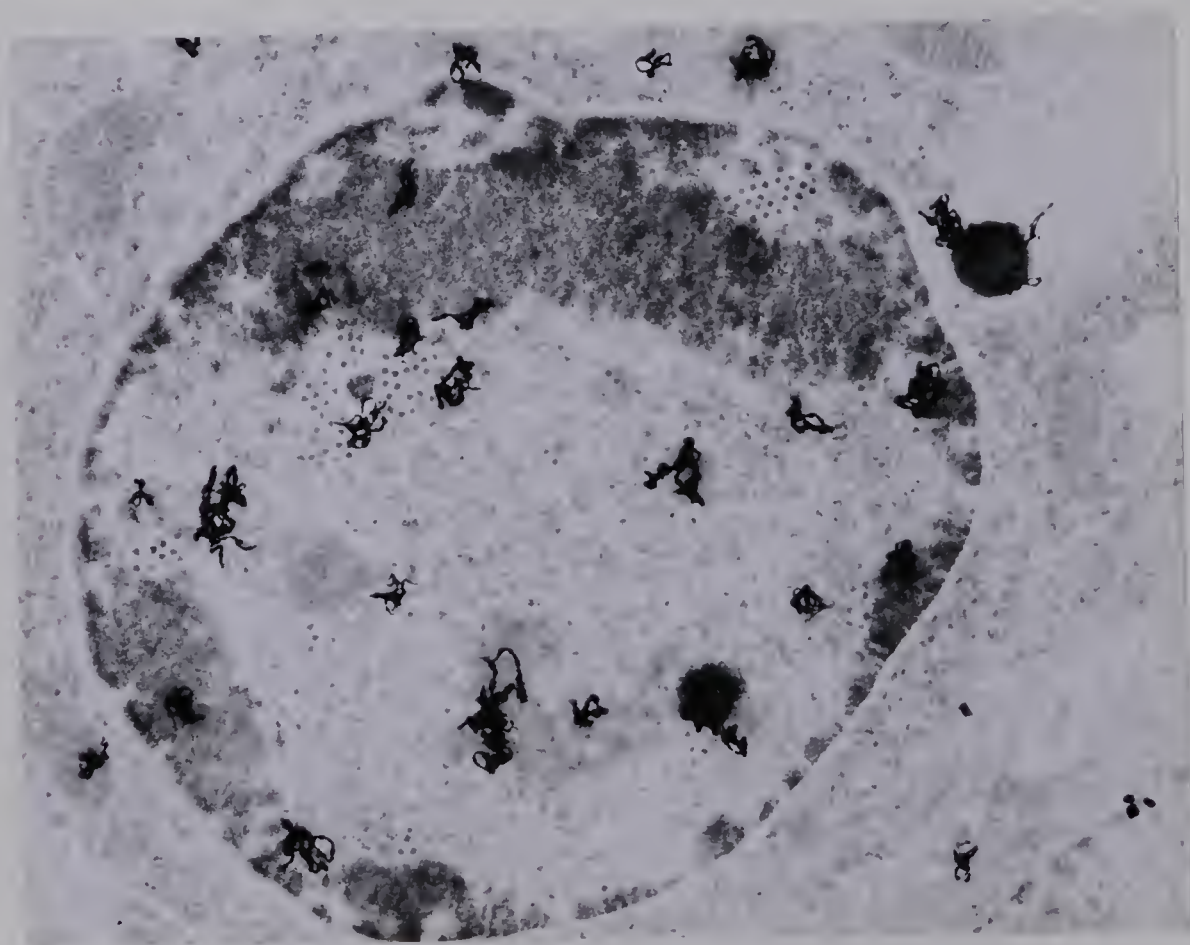
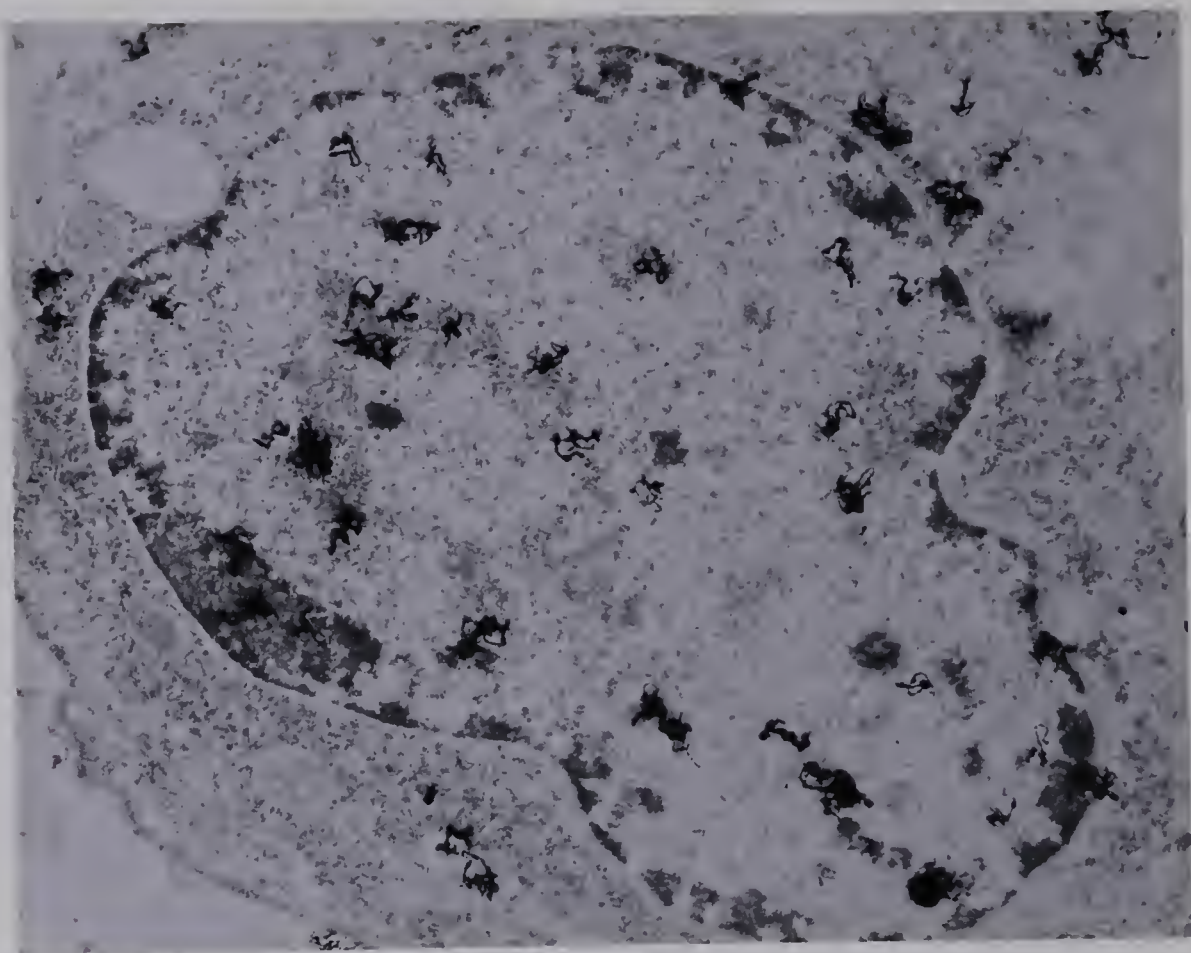


Fig. 49

Electron microscopic autoradiograph of MDC cell 16 hr after infection.

30 min pulse with tritiated leucine. The grains are located all over the nucleoplasm and cytoplasm but preferentially over the inclusions. X 12,000

Fig. 50

Electron microscopic autoradiography of MDC cell 18 hr after infection.

30 min pulse with tritiated leucine. Dark staining inclusion in the cytoplasm is labelled. A small inclusion (dark staining) in the nucleus is seen which is also labelled. X 12,500

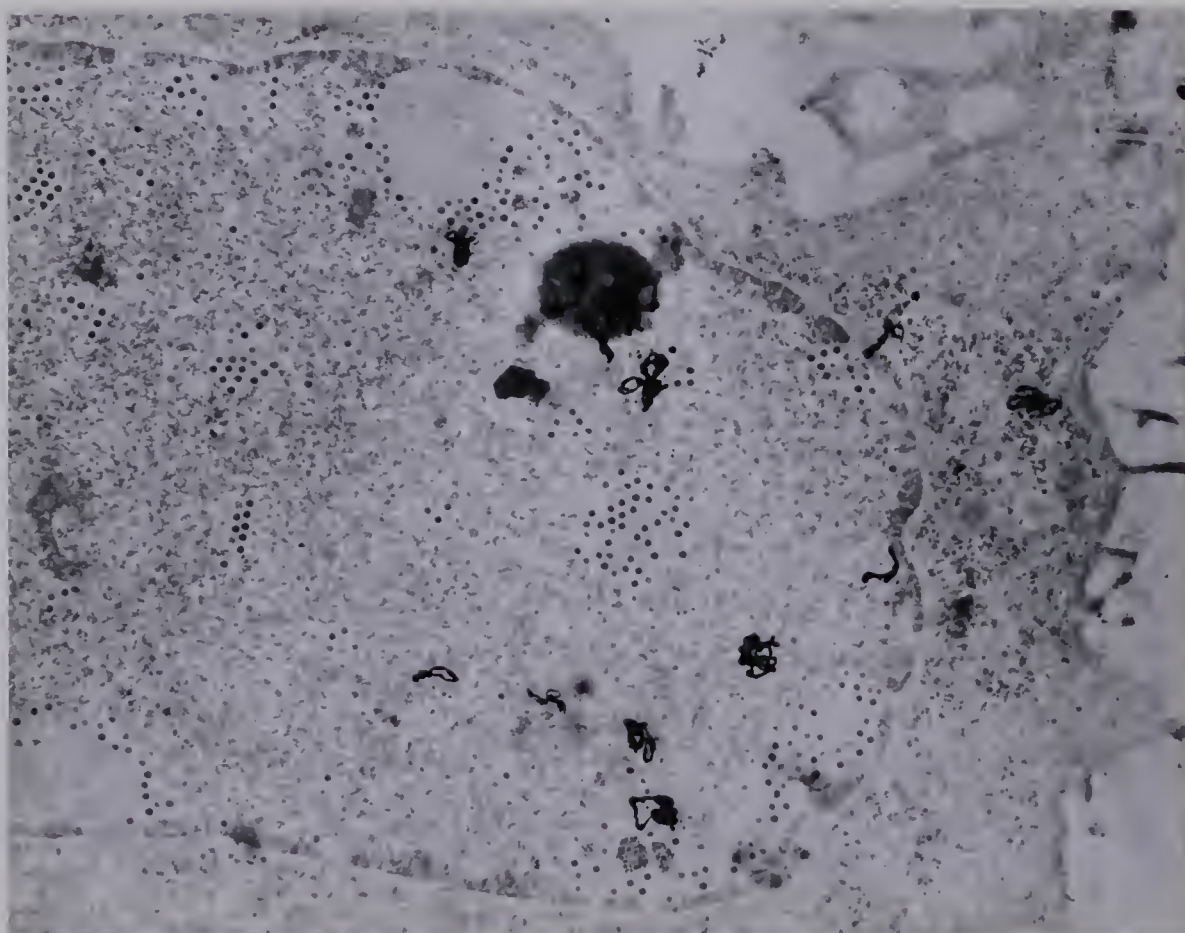
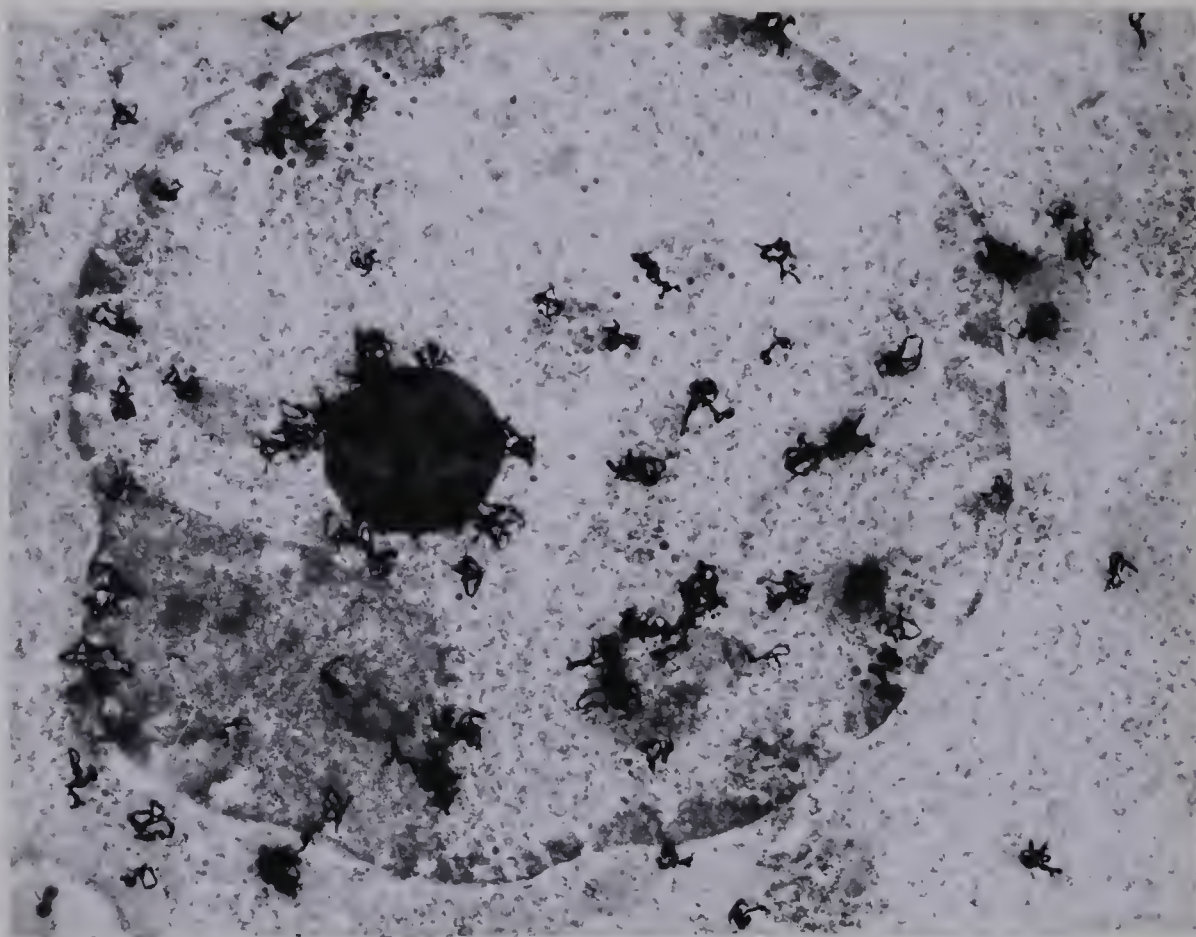


Fig. 51

Autoradiograph of infected MDC cell and labelled as in Fig. 50.

Dark staining inclusion in the nucleus is heavily labelled.
X 12,000

Fig. 52

Autoradiograph of infected MDC cell similarly treated to that in Fig. 50.

The light staining inclusions are not labelled.

Labelled dark staining inclusion is seen. X 11,500

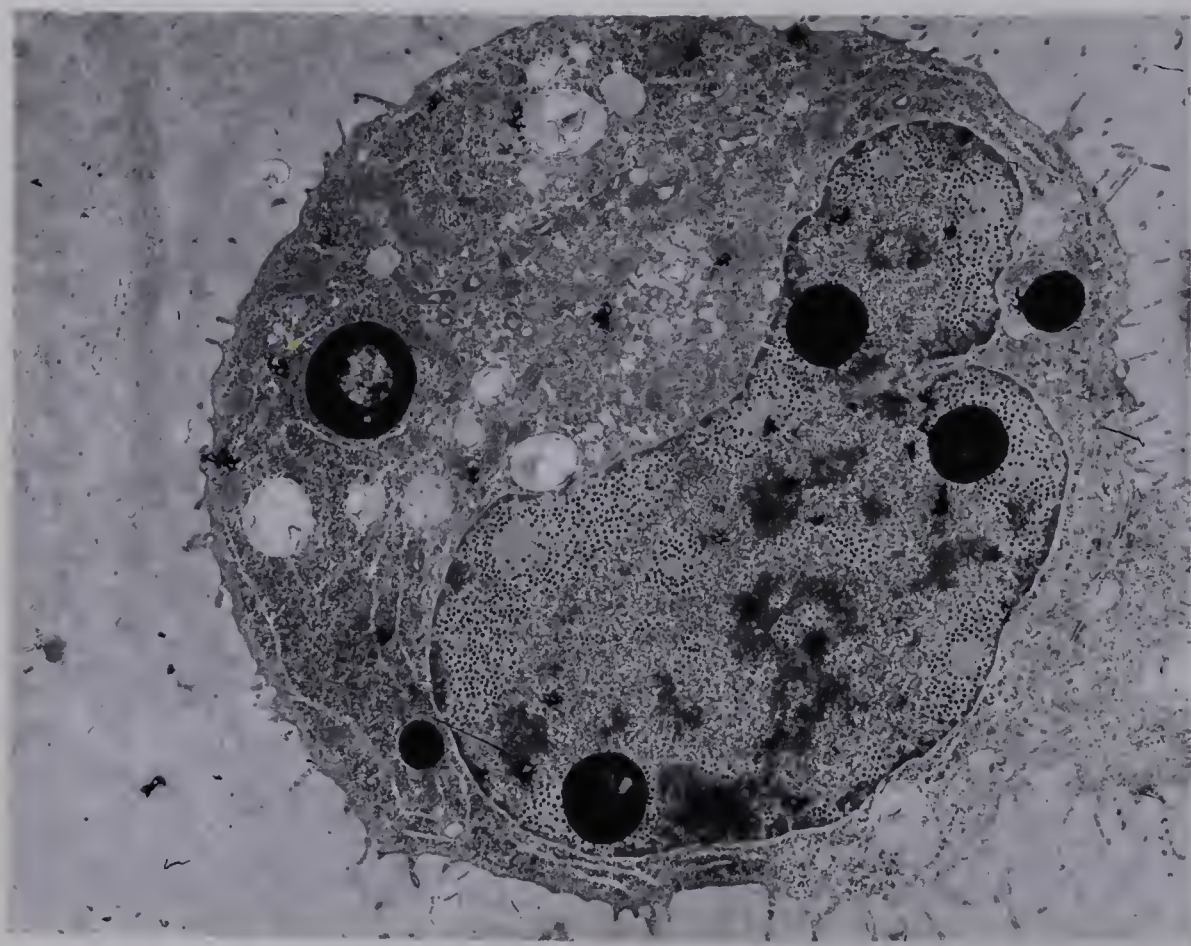
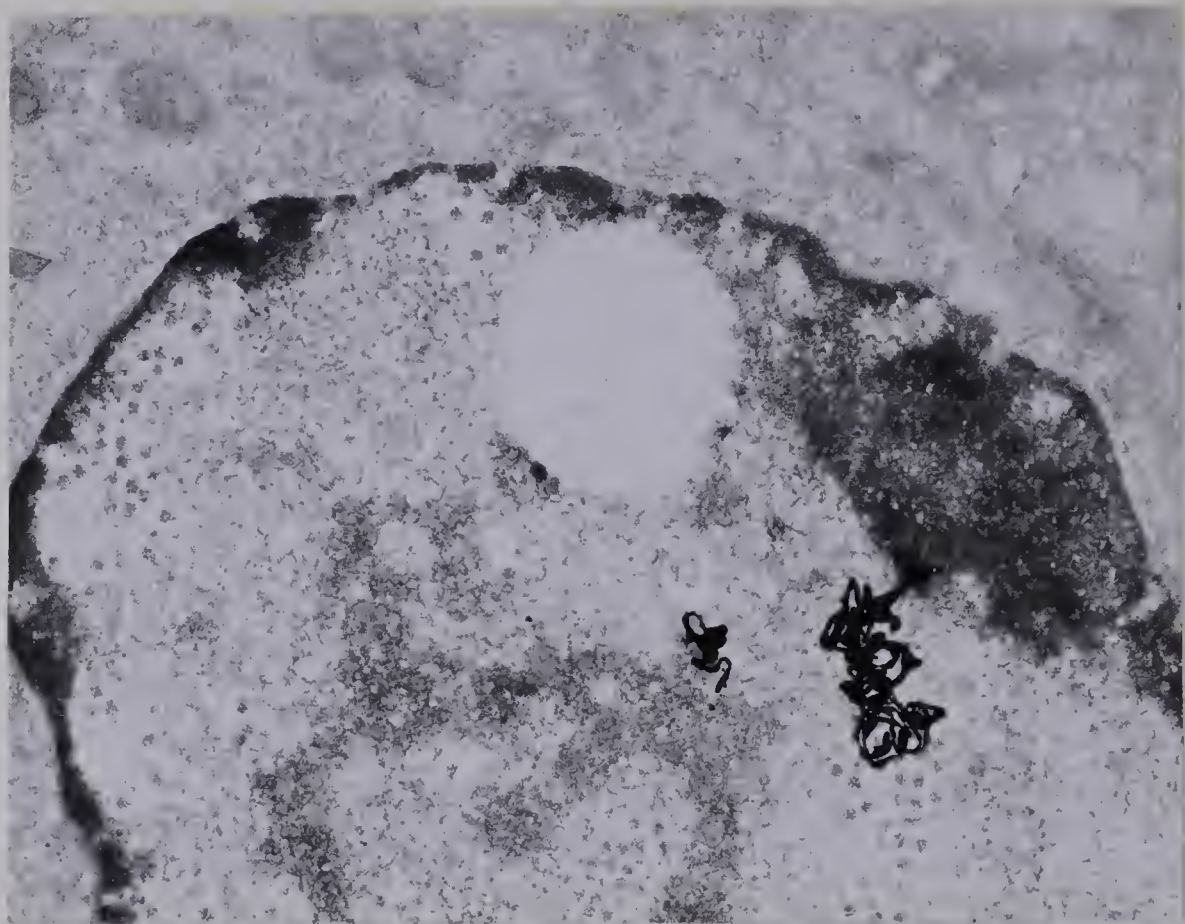


Fig. 53

Electron microscopic autoradiograph of MDC cell 18 hr after infection labelled with tritiated leucine for 30 min.

The section was floated on 0.25% solution of trypsin for 30 min then used for autoradiography. The radioactive material is completely removed from the digested dark staining inclusion (light circular area). X 18,000

Fig. 54

Electron microscopic autoradiograph of MDC cell 18 hr after infection.

The cell was labelled with tritiated leucine for 30 min then infected with ICL virus and incubated for 18 hr. None of the light and the dark staining inclusions either in the nucleus or in the cytoplasm are labelled. X 5,000

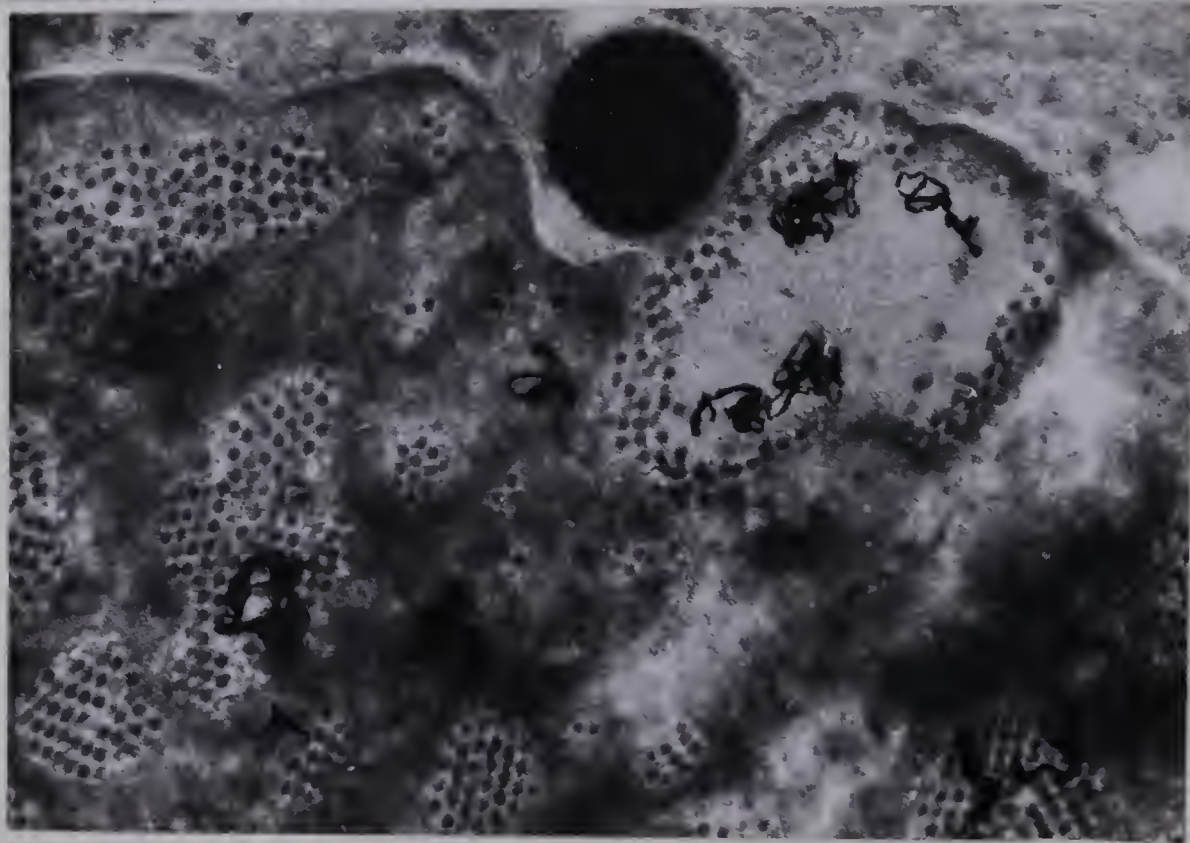
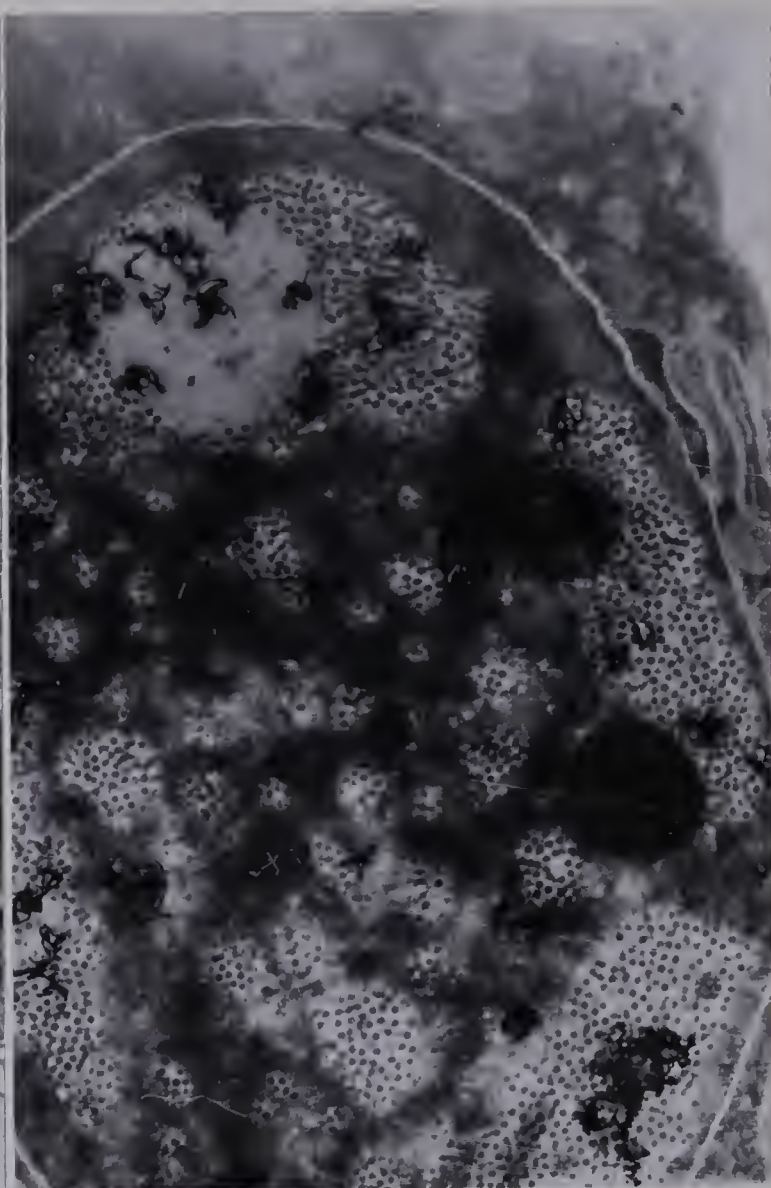
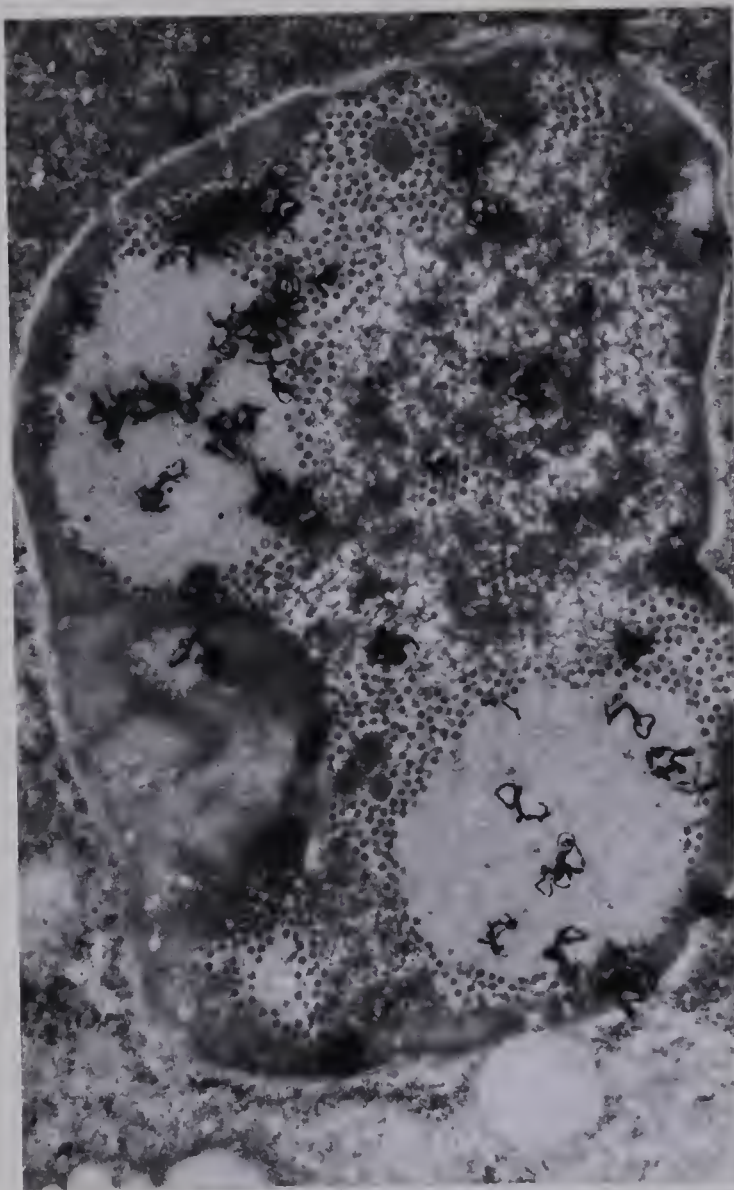


Fig. 55 (top left)

Electron microscopic autoradiograph of MDC cell labelled with tritiated leucine at 12 hr after infection for 30 min, then chased in fresh medium for 6 hr.
The light staining inclusions are labelled. X 13,500

Fig. 56 (top right)

Autoradiograph of infected MDC cell similarly labelled as in Fig. 55.
The dark staining inclusion in the nucleus is not labelled.
A labelled light staining inclusion is seen (at the top).
X 14,000

Fig. 57

Autoradiograph of infected MDC cell labelled with tritiated leucine as in Fig. 55.
Dark staining inclusion in the cytoplasm is not labelled.
The silver grains are seen over the light staining inclusion in the nucleus. X 25,000

DISCUSSION

The morphological changes occurring in host cells during the multiplication cycle of adenovirus have been studied with the light microscope (Boyer et al, 1957, Denny and Ginsberg, 1961) and with the electron microscope (Morgan et al, 1956, Harford et al, 1956, Morgan et al, 1960, Martinez-Palomo, 1967). These observations demonstrated the appearance of different inclusions in the nuclei of infected cells during the developmental process of virus. However, the origin of the inclusions and their role in virus maturation have not been clearly determined.

The sequential changes produced in primary dog kidney cells after infection with ICL virus have been studied with the electron microscope (Yamamoto, 1969) and were correlated with the lesions observed in the light microscope (Yamamoto, in press, Microbios). These observations demonstrated the formation of different types of inclusions, each appearing at a certain time after infection. It was also shown that, at the level of the light microscope, the alterations induced in ICL infected cells were different from those observed by Boyer et al (1957) in human adenovirus infected cells. At the electron microscopic level, the appearance of different inclusions in ICL virus infected cells did not resemble the inclusions found by Morgan et al (1960) in

adenovirus type 12 infected cells. These differences have been reviewed in detail in the Introduction.

The present study demonstrated that the morphological changes induced by ICL virus in a different kind of cell (MDC) were similar to those found by Yamamoto (1969) in primary dog kidney infected cells. The nature of these inclusions and their possible role in virus production will be discussed in relation to the results obtained from the enzyme, cytochemistry and autoradiography experiments.

1. The nature of the early and ring form inclusions

a) Composition: The earliest detectable change in the nucleus of ICL infected MDC cells was the appearance of granular dark staining inclusions. These inclusions which appeared at 12 hr post infection, were shown to be basophilic and Feulgen-positive when examined in the light microscope (Yamamoto, in press, Microbios). However, further information regarding the nature of these inclusions could not be obtained because of the difficulty of performing cytochemical studies on the infected cell embedded in Epon.

In this study, the use of water soluble embedding media (Leduc, 1967) made it possible to apply the enzyme digestion directly to the sections. Treatment of the infected cells with either pronase or trypsin resulted in the partial digestion of the

early inclusions and the reduction of their stain intensity which was due to the removal of proteins from these bodies. These inclusions which did not appear to be attacked by DNase before treatment with proteolytic enzymes, were readily removed when the DNase digestion was preceded by pronase or trypsin treatment. It seems that the early inclusions represented the association of protein material with DNA in a form that was not readily attacked by DNase. Moreover, the incorporation of radioactive thymidine into the early inclusions (Fig. 27) provided additional evidence of DNA content in such bodies.

The ring form inclusions which appeared in ICL infected cells by 16 hr post infection, morphologically, have been shown to be produced by the development of the early inclusions (Yamamoto, 1969). In this study, the direct relationship of the ring form structures to the early inclusions was confirmed by comparing their chemical composition. Proteolytic enzyme digestion of the ring form inclusions removed the protein content of these structures. They appeared with the same stain intensity as the early inclusions (Fig. 12, 14). Digestion with proteolytic enzymes followed by DNase resulted in the disappearance of these bodies. Furthermore, the incorporation of tritiated thymidine into these inclusions (Fig. 28)

was additional evidence to indicate the presence of DNA in these structures. It seems likely that the chemical composition of these two types of inclusions (early and ring form inclusions) is "nucleoprotein".

b) Nature of the DNA present in the inclusions:

The DNA existing in the inclusions may account, in part, for the excess of viral DNA found by Green (1962) in adenovirus type 2 infected cells. In the Results section, (Fig. 33, 34), it was shown that when the 12 hr infected cells were labelled with tritiated thymidine and then chased until the time of the appearance of virus particles, most of the radioactivity was found to be associated with the virus particles. These observations indicated that at least part of the labelled DNA present in the early inclusions was incorporated into the virus particles. It could be concluded that these inclusions were not simply the accumulation of excess DNA which was not used for the virus production, but at least part of it was functional DNA and participated in virus maturation.

Ginsberg et al (1967) reported that in adenovirus type 5 infected KB cells, the host DNA synthesis stopped at the time when viral DNA synthesis began. Recent studies by Hodge and Scharff (1969) on the multiplication of adenovirus type 2 in synchronized HeLa cells indicated that the initiation of viral

DNA synthesis during the G_1 , G_2 , and M phase in the life cycle of the cells resulted in the complete inhibition of host DNA synthesis. They also pointed out that the synthesis of viral DNA even during the period of active host DNA replication (S phase) caused a rapid decrease in the rate of host DNA synthesis. It can be assumed that, in most of the ICL infected cells, at the time of the beginning of the viral DNA synthesis which preceded the appearance of the early inclusions, the host DNA replication was arrested, and therefore the only DNA synthesized in the infected cells would be viral DNA. This assumption also agrees with the results obtained in this study which showed the incorporation of the labelled DNA present in the early inclusions, into the virus particles (Fig. 33, 34).

In 1967, Martinez-Palomo showed that the nucleoli of adenovirus type 12 infected KB cells were heavily labelled with radioactive thymidine. He concluded that the incorporation of thymidine into the nucleoli was due to the induction of host DNA synthesis which occurred after infection. However, this rapid incorporation of tritiated thymidine into the nucleoli was not observed in ICL virus infected cells.

In general, the synthesis of macromolecules in the cells infected with oncogenic DNA viruses (including adenovirus type 12) is different from

the other DNA virus infected cells. They usually cause an increase in the rate of host DNA synthesis. These observations have been reported for other nuclear DNA viruses, such as polyoma (Dulbecco et al, 1965) and SV 40 (Kit et al, 1967) which caused the induction of host DNA replication.

Electron microscope autoradiography by Comingo and Kakefada (1968) revealed that the synthesis of cell DNA in synchronized human amnion cells was associated with the nuclear membrane. In our experiments, some of the tritiated thymidine labelled cells showed the location of a few silver grains over the nuclear membrane. This could possibly be due to the continuous host DNA synthesis in some of the infected cells in which the viral DNA replication had started after the onset of S phase (Hodge and Scharff, 1969).

c) The nature of the proteins present in the early and ring form inclusions:

In addition to the proteolytic enzyme digestion, the incorporation of radioactive leucine into the early and ring form inclusions confirmed the evidence of the presence of protein in these bodies. The nature of this protein(s) will be discussed in relation to the studies of other investigators on the multiplication of human adenoviruses.

In 1966, Kalnins et al observed the appearance

of different inclusions in the nuclei of human amniotic infected cells with adenovirus type 12. A type of inclusion which appeared with a fibro-granular structure, was stained by ferritin-labelled antibodies to viral antigens, indicating its association with viral antigenic material. Further studies by Martinez-Palomo (1967) demonstrated that these bodies were composed of nucleoproteins.

Although these inclusions appeared at a late stage of infection (24 hr after infection), in respect to their texture and chemical composition, they had some similarity to the early and the ring form inclusions formed in ICL infected cells. It is probable that the protein content of the ICL virus induced inclusions may also be virus antigenic material. Furthermore, Wilcox and Ginsberg (1963) reported that the viral protein synthesis in adenovirus type 5 infected HeLa cells started shortly after the initiation of viral DNA synthesis which was about 11 hr after infection. Similar observations have been made by White et al (1969) in adenovirus type 2 infected HeLa cells. The immunological studies by Hayashi and Russell (1968) on the development of adenovirus types 5 and 7 antigen in human embryo kidney cells, showed that the viral antigen was detectable in the nuclei of host cells at 9 hr after infection.

It is tempting to speculate that in ICL virus infected cells, the protein synthesized during the early stage of infection which was incorporated into the inclusions was viral protein. Further immunological studies are required to prove this assumption and to determine whether this protein(s) contains one or more particular adenovirus antigens.

d) Presence of RNA in the inclusions:

The undetectable changes in the ultrastructural appearance of the early and the ring form inclusions after digestion with RNase, may lead to the conclusions that: (a) the absence of RNA in these inclusions; (b) the failure of RNase to attack RNA containing material; (c) association of RNA with the inclusion in such a way that its removal (by RNase) did not change the ultrastructural appearance of these bodies. However, autoradiography experiments on the infected cells labelled with tritiated uridine showed that, in fact, the third conclusion was true. Exposure of the infected cells to radioactive uridine revealed the incorporation of this precursor into the nucleoli and into both the early and ring form inclusions (Fig. 41, 43). Digestion of the thin sections of uridine labelled cells with RNase resulted in the removal of radioactive material from the nucleoli and inclusions. Sensitivity of the labelled material to digestion with RNase indicated that the reduced

silver grains located over the inclusions was due to the association of radioactive RNA within these bodies. Likewise, it was shown (Table II) that about 80% of the radioactive uridine taken up by the infected cells was incorporated into RNA. Only 10% of the radioactivity was found in DNA, which means that one out of 10 grains located over the nuclei of uridine labelled cells could be due to the presence of radioactive DNA. This amount of ³H-uridine incorporated into DNA did not interfere with the interpretation of the results obtained from the radioactive uridine labelled cells, particularly from the cells labelled for a short time (10 min) in which the number of reduced silver grains located over the nuclear area was quite limited.

Histochemical studies by Mayor (1964) on the multiplication of adenovirus types 3 and 7 in monkey kidney cells, showed the presence of a labile RNA in the nuclei of infected cells. Using acridine orange staining technique to differentiate the various types of nucleic acids, she showed the presence of bright rings of RNA staining material surrounding the DNA containing inclusions in the nuclei of 20 hr infected cells. However, the nature of this RNA staining material which was observed at a maximum at 24 hr after infection was not discussed.

An increase of RNA synthesis in adenovirus infected cells which was due to the formation of viral mRNA has been reported by several investigators (Flanagan and Ginsberg, 1964, Rose et al, 1965, Thomas and Green, 1966). The high incorporation of uridine in ICL virus infected cells (Table II) could be due to the increase of RNA synthesis after infection. Moreover, White et al (1969) showed that the viral mRNA produced in adenovirus type 2 infected HeLa cells was relatively stable with a functional half life of about 6 hr. In ICL virus infected cells, most of the radioactivity incorporated into the cells as uridine was associated with the nucleoli and with the early inclusions. The incorporation of tritiated uridine into the nucleoli agrees with the results obtained by Penman et al (1966) who showed the synthesis of ribosomal RNA in these organelles, but the nature of the RNA associated with the inclusions is not clear.

Several possibilities can be assumed for the origin of the RNA present in the inclusions:

- (i) it was synthesized in the nucleoli and then moved into the inclusions
- (ii) it was directly synthesized in the inclusions
- (iii) this RNA was copied from the viral DNA present in the nucleoplasm, then gathered around the inclusions.

Since in infected cells labelled with tritiated uridine for a short time (10 min), the radioactivity was mostly associated with the inclusions (Fig. 42, 43), it seems more likely that in fact the early and ring form inclusions were the site of RNA synthesis. The presence of viral DNA in these bodies which was demonstrated before (Fig. 33, 34) leads to the speculation that the RNA associated with the inclusions was viral mRNA which was copied directly from the virus specific DNA.

2. The nature of the dark and light staining inclusions

The appearance of dark staining inclusions in the nuclei of primary dog kidney cells infected with ICL virus was shown by Yamamoto (1969). In MDC cells, however, these inclusions which appeared 18 hr after ICL virus infection, were observed both in the nuclei and in the cytoplasm. Digestion of infected cells in thin sections with proteolytic enzymes resulted in the complete removal of these structures. Sensitivity to digestion with proteolytic enzymes and the incorporation of radioactive leucine into these bodies suggested that they were composed of protein(s). In addition, the non-incorporation of radioactive uridine and thymidine into the dark staining inclusions during the time of formation indicated the lack of nucleic acids in these bodies.

Morgan et al (1957, 1960) demonstrated the presence of paracrystalline structures in cells infected with

adenovirus type 5. These crystals which were shown to be protein, did not stain with fluorescent antibody to viral antigens, indicating the non-structural virus nature of the crystals. The same structures were observed by Weber and Stich (1969) in Hep 2 cells infected with adenovirus type 2 strain. However, the fine structure of these crystals was completely different from the dark staining inclusions observed in ICL virus infected cells, since the crystals were composed of cylindrical tubules arranged in parallel, whereas the dark staining inclusions in ICL infected cells had a tightly granular structure.

Martinez-Palomo (1967) observed the appearance of dark staining inclusions in KB cells infected with adenovirus type 12. These inclusions in respect to the stain intensity and sensitivity to digestion with proteolytic enzymes, resembled the dark staining inclusions found in ICL infected cells, but in respect to the time of appearance were quite different. The adenovirus type 12 inclusions were observed at the early stage of infection before the appearance of virus particles, whereas the dark inclusions in ICL infected cells appeared at the late stage of infection, after the formation of a large number of virus particles.

The light staining inclusions which appeared simultaneously with the dark inclusions in the nuclei of ICL virus infected cells were not attacked by nucleases. The fact that they were readily digestible by proteolytic

enzymes, demonstrated that these inclusions were protein in nature.

In this study we found that the late inclusions (dark and light staining inclusions) which could be seen frequently in thin sections of infected cells at 20 hr after infection, appeared at 18 hr post infection in some of the infected cells. We considered 18 hr post infection the time of formation of these inclusions. When the infected cells were labelled with radioactive leucine at 18 hr post infection, the dark staining inclusions were heavily labelled. On the other hand, when the 12 hr infected cells were labelled with tritiated leucine, and then chased until the appearance of the late inclusions, no radioactivity could be detected in the dark staining inclusions. These results lead to the conclusions that the dark staining inclusions were not formed from the accumulation of presynthesized proteins, but they were synthesized at the time of formation.

The light staining inclusions which were susceptible to digestion by proteolytic enzymes, did not incorporate radioactive leucine at 18 hr after infection (the time of appearance of these inclusions). However, when the cells were labelled with tritiated leucine at 12 hr after infection and then chased for 6 hr, these inclusions were labelled. It could be concluded that part of the proteins synthesized during the infection, before the appearance of virus particles, accumulated, and appeared as light staining inclusions.

The nature of the proteins present in the late inclusions and whether they contain viral structural proteins remains to be determined.

The undetectable incorporation of radioactive uridine and thymidine into the light staining inclusions indicated the absence of nucleic acid in these structures. The significant observation of the incorporation of radioactive uridine into the nucleoli of infected cells at the late stage of infection (18 hr post infection) suggested that continuous synthesis of RNA occurred in these organelles (Fig. 44), even during the period of active virus synthesis.

Ginsberg et al (1967) reported that the host protein synthesis in adenovirus type 5 infected KB cells was inhibited by 20 hr post infection. They suggested that the inhibition of host protein synthesis in infected cells could be due to the competition for free ribosomes between host and viral mRNA. The same suggestion has been made by White et al (1969) for the inhibition of host protein synthesis in HeLa cells infected with type 2 adenovirus. If these suggestions are true in ICL virus infected cells, it may be assumed that the continuous RNA synthesis in the nucleoli, even at the late stage of infection, was due to the production of ribosomal RNA. This RNA was required for the formation of ribosomes to be used by viral mRNA for protein synthesis.

Margination of host DNA

Margination of the nuclear chromatin was described in cells infected with herpes simplex virus by Crouse et al (1950) and herpes B virus by Reissig and Melnick (1955). Similar observations were reported in infected cells with pseudorabies virus by Kaplan and Ben-Porat (1963). However, these observations were based on light microscopic examination of the virus infected cells and the resolution was not adequate to show clearly the non-participation of host DNA in the virus maturation.

By electron microscope autoradiography technique, we showed that prelabelled DNA of the host cells infected with ICL virus marginated along the nuclear membrane (Fig. 37, 38). These experiments indicated that:

1. Apparently, host DNA does not participate in the formation of viral induced inclusions.
2. There is no degradation and incorporation of host DNA into the virus particles.

The fact that margination of cellular DNA occurs relatively early in the infective process suggests that it may have an active role in the metabolic changes which take place in the cell after virus infection.

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